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A COMPARATIVE STUDY OF TRIOSEPHOSPHATE ISOMERASE

by



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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled A COMPARATIVE STUDY OF TRIOSEPHOSPHATE ISOMERASE submitted by Peter K. Chiang in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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I owe much to my wife, Sabrina, for her encouragement and help.

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ABSTRACT

Triosephosphate isomerase has been purified from thoracic muscle of the mosquito, *Aedes aegypti* (L.), and the housefly, *Musca domestica* (L.). The estimated molecular weight for triosephosphate isomerase from the housefly, mosquito and rabbit is 60,000. Both the housefly and rabbit triosephosphate isomerase are electrophoretically cationic at pH 6.5 and 8.0. The pH optimum for the mosquito and housefly triosephosphate isomerase is about pH 8.8. The activity of triosephosphate isomerase from leg muscle of the cockroach, *Periplaneta americana* (L.), is about twice that of thoracic muscle.

With dihydroxyacetone phosphate as substrate, the triosephosphate isomerase activity exhibits sigmoidal kinetics, and can be approximated by assuming a second order Michaelis-Menton equation. The apparent K_m for the mosquito triosephosphate isomerase does not change from 25 C to 40 C. With glyceraldehyde-3-phosphate as substrate, the isomerase activity, except for the occurrence of substrate inhibition, can be described by the simple Michaelis-Menton equation.

Triosephosphate isomerase is inhibited by: inorganic phosphate, DL- α -glycerophosphate, β -glycerophosphate, 3-phosphoglycerate, ribose-5-phosphate, ADP, ATP, CTP, CDP, UTP, GTP, citrate, oxaloacetate, succinate, folic acid, 1-hydroxy-3-iodo-2-propanone phosphate, and arsenate. Cooperativity among ligands exists, and conformational change in the enzyme may take place. Both pH and temperature have profound effects on the inhibition of mosquito triosephosphate isomerase by inorganic phosphate. It is postulated that triosephosphate isomerase is a regulatory enzyme with an allosteric site.

AUTOBIOGRAPHICAL SKETCH

I was born in Hong Kong on October 20, 1941. I attended the Raimondi College for my high school education.

I graduated in 1965 from the University of San Francisco with a B. Sc. in biology. I was on the honor roll for the academic years of 1963 and 1964, and was awarded a scholarship.

After I received an M. Sc. in entomology at the University of Alberta in 1967, I became interested in insect flight muscle biochemistry.

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INTRODUCTION

Triosephosphate isomerase (TPI) catalyzes the interconversion of glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP). Chefurka (1954) demonstrated TPI in homogenates of the housefly. TPI is extremely active in the housefly sarcoplasm (Sacktor and Cochran, 1957), and TPI is always the most active enzyme among the constant-proportion group of extramitochondrial, glycolytic enzymes of insects and vertebrates (Pette, Luh and Bücher, 1962; Vogell *et al.*, 1959). The activity of TPI increased with the rest of the constant-proportion group in the developing flight muscle of the locust, and reached its peak eight days after molting (Brosemer, Vogell and Bücher, 1963). TPI is localized in cross-striated muscles at the site of isotropic zones (Sigel and Pette, 1969).

TPI has been crystallized from calf muscle (Meyer-Arendt, Beisenherz and Bücher, 1953; Beisenherz, 1955), rabbit muscle (Czok and Bücher, 1960), bovine lens (Burton and Waley, 1968b), and chicken muscle (Wolfenden, 1969). Regardless of the origin of tissues, TPI could be resolved into 3 bands electrophoretically (Burton and Waley, 1966, 1968b; Kaplan *et al.*, 1968; Scopes, 1964). After digestion by trypsin, the existence of 2 subunits of TPI was suggested by the number of peptides present (Burton and Waley, 1966). X-ray crystallography with rabbit muscle TPI confirmed that there are 2 subunits, each with a molecular weight of $26,203 \pm 3000$; but by equilibrium ultracentrifugation, a molecular weight of 60,000 was obtained (Johnson and Waley, 1967). The enzyme is a dimer in which the 2 subunits are related by a 2-fold rotation axis. The 3 electrophoretic

bands might have been formed by the interaction of the 2 subunits (A and B) to give: AA, AB, and BB (Kaplan *et al.*, 1968). Mitochondrial TPI has been demonstrated from rat liver and kidney (Box and Shonk, 1960).

Bloom and Topper (1956) suggested that an enediol intermediate was involved in the isomerase reaction. This was supported by the findings of Rieder and Rose (1959) who showed that the interconversion of G-3-P and DHAP in tritiated water by TPI resulted in the formation of monotrhitated DHAP. Although G-3-P exists in 2 forms, the geminal diol and the free aldehyde, it was the aldehyde form of G-3-P that was liberated by TPI (Trentham, McMurray and Pogson, 1969).

Reaction of TPI with iodoacetate took place in 2 phases: first, at pH 6.3, cysteine and methionine groups reacted and enzymic activity was unimpaired; finally, histidine reacted, and enzymic activity was lost (Burton and Waley, 1966). Photo-oxidation of TPI lead to inactivation, with loss of cysteine, histidine and tryptophan, but little loss of tyrosine; histidine was postulated as the basic group at the active site (Burton and Waley, 1966).

Recent publications indicate that TPI is perhaps a regulatory enzyme. An allosteric site was suggested for rabbit TPI (Snyder and Lee, 1966). The evidence for 2 binding sites per TPI molecule was further substantiated by the demonstration that 1.7 moles of 1-hydroxy-3-iodo-2-propanone phosphate (IAP), an active-site-directed irreversible inhibitor, were bound per mole of rabbit TPI (Hartman, 1968). CAP, the chloro analog of IAP, esterifies an essential glutamic acid residue at the active site of TPI (Hartman, 1970a).

By computer simulation of beef heart glycolysis, Achs and Garfinkel (1968) were puzzled by the failure of the ratio of DHAP/G-3-P to achieve equilibrium. The nonequilibration of DHAP/G-3-P was also noted in the rat liver *in vivo* (Rose *et al.*, 1962; Veech *et al.*, 1969). The equilibrium constant of DHAP/G-3-P was observed as 20-25 (Meyerhof and Junocwicz-Kocholaty, 1943), 28 (Lowry and Passonneau, 1964), and 22 (Beisenherz, 1955; Burton and Waley, 1968a; Veech *et al.*, 1969). With G-3-P as substrate, TPI had a turnover number of about 1,000,000 at 38 C (Meyerhof and Beck, 1944).

STATEMENT OF THE PROBLEM

The role of TPI in insects, particularly in metabolism in flight muscle, has not yet been explored. But its importance by the strategic position it occupies, linking together the α -glycerophosphate (glycero-P) shunt, the glycolytic pathway and the pentose shunt, cannot be overlooked. For metabolism in general, the G-3-P formed from DHAP, other than being an intermediate in glycolysis, can participate in the pentose shunt, toward the synthesis of glucose-6-phosphate, to generate NADPH for the reduction of dihydrofolic acid to tetrahydrofolic acid, and for the synthesis of fatty acids. The glycero-P formed by DHAP participates directly in the glycero-P shunt, the sole NAD generating mechanism for insects that use carbohydrates for flight (Chefurka, 1965; Sacktor, 1965), or can lead to the formation of glycerol and glycerides. TPI is one of the metabolic wheels mentioned by Racker (1965).

It is my purpose:

- (1) To isolate and purify TPI from the flight muscle of the yellow fever mosquito, *Aedes aegypti* (L.), and the housefly, *Musca domestica* (L).
- (2) To study and compare the properties of TPI from these insects and the rabbit.
- (3) To elucidate the role TPI plays in metabolism, especially in insect flight muscle.

MATERIALS AND METHODS

Materials

The mosquito *A. aegypti*, housefly *M. domestica*, and the cockroach *Periplaneta americana* (L.) were reared in an insectary at 24-27 C, and 40% relative humidity. The mosquito larvae were fed rabbit pellets, and the adults were fed 5% sucrose solution, and rat blood. Blood meals were withheld from the experimental mosquitoes. The housefly larvae were fed bran soaked in milk, and the adults were fed with 5% sucrose solution. The cockroaches were supplied rabbit pellets and water. The adults of these insects were killed by chilling and stored in a freezer.

Glyceraldehyde-3-phosphate dehydrogenase, α -glycerophosphate dehydrogenase, DHAP, G-3-P, and rabbit muscle TPI were purchased from Sigma Chem. Co., ^{32}P from New England Nuclear; NADH and NAD from Calbiochem Co. IAP was a gift of Dr. F. C. Hartman, Oak Ridge National Laboratory, Tenn. The rest of the reagents and chemicals were of the highest purity, obtained mainly from Sigma Chem. Co., Calbiochem., Fisher Scientific, and Pharmacia.

Methods

Enzyme assays

All assays were done in duplicate or triplicate, at 30 C, or as stated.

Conversion of DHAP to G-3-P

Coupling enzyme method: The assay for TPI activity with DHAP as substrate was based on the method of Marquardt, Carlson and Brosemer (1968) for assaying glyceraldehyde-3-phosphate dehydrogenase. The reaction mixture consisted of 100 mM triethanolamine (TEA), 28.8 mM mercaptoethanol, 0.3 mM NAD, 13 mM arsenate, and 20 μ g of rabbit glyceraldehyde-3-phosphate dehydrogenase. DHAP was added first, and after 5 min of incubation, the reaction was started by the addition of TPI. The final volume was 1 ml (pH 8.0 for mosquito TPI; pH 8.8 for housefly and rabbit TPI). Readings were taken every min for 3 min.

Modified assay method: Since it was demonstrated that arsenate, a necessary ingredient in the coupling enzyme method, could inhibit rabbit TPI, the following modified assay method was used: The assay solution consisted of 100 mM TEA, 5 mM EDTA, and variable amounts of DHAP. The final volume was 1 ml (pH 8.0 for mosquito TPI; pH 8.8 for housefly and rabbit TPI, or as indicated). TPI was added to the solution to initiate the reaction. After 2 or 3 min of incubation, the reaction was stopped by the addition of 0.4 ml of 5% TCA. The pH of the assay solution was then neutralized by the addition of one drop of 2.5 N NaOH. The volume was made up to 1.5 ml. In order to assay the amount of G-3-P formed, 0.5 ml of the solution was pipetted into a cuvette containing 0.5 ml of 50 mM TEA, 14.4 mM mercaptoethanol, 13 mM arsenate, and 0.6 mM NAD (pH 8.2), and 20 μ g of rabbit glyceraldehyde-3-phosphate dehydrogenase was then added. The reduction of

NAD, which is usually complete within 5 min, was read at 340 nm in a Beckman DU-2 spectrophotometer. The blank contained the same mixture, except glyceraldehyde-3-phosphate dehydrogenase was omitted. It was checked that this assay procedure did not produce any interfering substance, and that the zero time control did not give any net reading.

Conversion of G-3-P to DHAP

Enzyme activity was measured by the rate of change of NADH concentration at 340 nm. The cuvette contained 50 mM TEA, 5 mM EDTA, 0.18 mM NADH, 20 μ g of rabbit α -glycerophosphate dehydrogenase (pH 8.8). The G-3-P was added first, and the reaction initiated by the addition of TPI. The final volume was 1 ml. Readings were taken every min for 3 min. The blank contained all the ingredients except TPI.

One unit of enzyme activity is defined as the amount of enzyme catalyzing the disappearance of 1 μ mole of substrate per min under the assay condition. The extinction coefficient was taken as 6.22×10^3 for NADH.

Protein concentrations were determined either by U.V. absorption (Layne, 1957), or by the method of Lowry *et al.* (1951).

Equilibrium dialysis with radioactive inorganic phosphate (Pi)

Equilibrium dialysis was performed according to the method of Changeux, Gerhart and Schachman (1968). Rabbit TPI was dialyzed in 2 changes of buffer overnight: 50 mM TEA, 2.5 mM EDTA, pH 8.0, 4 C. One ml of TPI solution (2.5 mg) was placed in one compartment,

and one ml of the radioactive Pi in the other compartment of the dialysis cell (Chemical Rubber Co.). The dialysis membranes (Union Carbide) were pre-soaked in the same dialysis buffer. The cells were placed on a mechanical shaker in a cold room, 2 C. After 24 hr, 20 λ of the solution were removed from both compartments and each mixed with 1 ml of distilled water. This is based on Cerenkov radiation for high energy β -emitters that can use distilled water as the counting medium (Haviland and Bieber, 1970). The total ligand (Pi) concentration was varied from one cell to another, but the amount of radioactivity of ^{32}P was maintained at about 4×10^5 cpm/20 λ . Samples were counted with a Picker Nuclear/Liquimat 220 scintillation counter with output on a Digital PD P-8/L computer. The window widths for channel A and B were determined from spectra analyzed by a Nuclear Chicago multi-channel analyzer.

Electrophoresis

Electrophoresis was done on cellulose acetate strips in a Gelman Delux Electrophoresis chamber at 4 C. The buffer used was 10 mM Tris, 0.4 mM EDTA, pH 8.0 and pH 6.5. The strips were stained with Ponceau S. TPI could be eluted from the strips by soaking in 10 mM Tris, 0.4 mM EDTA (pH 7.8).

Computer analysis of data

All the kinetic data were analyzed by programs written in APL\360 (Appendix), and processed by an IBM 360/67 computer via an IBM 2741 remote terminal. Some of the graphs presented here were

drawn by the Calcomp plotter with programs written in Fortran IV.

All the programs are written by the method of least squares.

The Wilkinson's plot was programmed according to the equation:

$$S/V = K_m/V_m + S/V_m \quad (1)$$

where S is the substrate concentration, V the velocity, K_m the Michaelis-Menton constant, and V_m the maximal velocity. The 95 percent confidence limits for K_m were determined by Fieller's theorem (1954). The Hill plot was programmed according to the Hill equation:

$$\text{Log } \frac{V}{V_m - V} = N \text{ Log } S - \text{Log } K \quad (2)$$

Depending upon situations, the Hill coefficient (N), or the slope, can either be interpreted as the number of substrate binding sites on the enzyme molecule, or the site-site interaction coefficient (cooperativity) for an enzyme (Atkinson, 1966; Monod, Wyman and Changeux, 1965; Sanwal and Cook, 1966). K has a different meaning from the ordinary K_m , except the apparent K_m or $S_{0.5}$ can be estimated when the left-hand side of the equation is equal to zero (Atkinson, 1966).

As for the estimation of the order of binding of an inhibitor on an enzyme molecule, Loftfield and Eigner (1969) expanded the equation by Taketa and Pogell (1965) into the following equations:

$$\text{Log } V_0 + \text{Log} \left(\frac{1}{V} - \frac{1}{V_0} \right) = N \text{ Log } I + \text{Log} \frac{K_m}{K_i (K_m + S)} \quad (3)$$

and

$$\text{Log } V_0 + \text{Log} \left(\frac{1}{V} - \frac{1}{V_0} \right) = N \text{ Log } I + \text{Log} \frac{1}{K_i} \quad (4)$$

where V is the inhibited rate, V_0 the uninhibited rate, N (slope) is the interaction coefficient or the apparent number of inhibitor binding sites per enzyme molecule, I the concentration of the inhibitor, S the substrate concentration, K_m the Michaelis-Menton constant, K_i the dissociation constant of the enzyme-inhibitor complex. Equation 3 is for competitive inhibition, and equation 4 for non-competitive inhibition.

But equation 3 and 4 can further be simplified into:

$$\text{Log } \frac{V_0 - V}{V} = N \text{ Log } I + \text{Log } \frac{K_m}{K_i (K_m + S)} \quad (5)$$

$$\text{Log } \frac{V_0 - V}{V} = N \text{ Log } I + \text{Log } \frac{1}{K_i} \quad (6)$$

respectively.

An inhibitor may act on both V_m and K_m , giving a mixture of competitive and non-competitive effects, and can be described by the following equation (Dixon and Webb, 1965):

$$V = \frac{V_m}{1 + \frac{K_m}{S} + \frac{I}{K_i} \left(\frac{K_m}{S} + \frac{K_m}{K'_m} \right)} \quad (7)$$

where V_m is the uninhibited rate at maximum substrate concentrations, K'_m the dissociation constant of enzyme-inhibitor-substrate complex, and the rest as defined before. Equation 7 can be transformed into:

$$\text{Log } \frac{V_0 - V}{V} = N \text{ Log } I + \text{Log } \frac{K_m (K'_m + S)}{K_i K'_m (K_m + S)} \quad (8)$$

For equations 5, 6, 7 and 8, a plotting of $\text{Log } ((V_0 - V)/V)$ against $\text{Log } I$ should yield a straight line, the slope of which equals N . In each case, I_{50} (concentration of inhibitor that gives 50 percent inhibition) is found at that value of I where the left-hand side of the equation is equal to zero.

From the Dixon plot, K_i and pattern of inhibition were determined by an algorithm method. Equilibrium dialysis was analyzed by the Scatchard plot (Scatchard, 1949) which was programmed according to the equation:

$$r/c = k(n - r) \quad (9)$$

where r is the molar ratio of ligand bound to the protein, c the unbound ligand concentration, k the intrinsic association constant, n the number of binding sites per mole of protein.

EXPERIMENT SECTION

Purification of Housefly Triosephosphate Isomerase

All the steps were carried out at 0-4 C, and Tris-EDTA buffers were used throughout the purification. The enzyme activity was assayed with G-3-P as substrate.

1. Extraction: Fly thoraces, 3.5 gram, were homogenized by a Sorval omni-mixer for 1 min with 21 ml of 0.3 M sucrose, 10 mM EDTA, 0.1 M Tris (pH 7.8), and centrifuged at 10,000 x g for 45 min. The supernatant was filtered through cheese cloth. The residue was homogenized again with 10 ml of the extraction buffer, and centrifuged again.
2. Ammonium sulfate fractionation: Powdered ammonium sulfate was added to the combined supernatants to a final concentration of 2.6 M over a 3 hr period. The suspension was centrifuged at 10,000 x g for 45 min, and the residue discarded.
3. DEAE cellulose chromatography: The supernatant was dialyzed by the continuous flow technique of Schulz, Gazith and Gooding (1967), using 5 mM Tris, and 2 mM EDTA buffer (pH 7.8). The DEAE cellulose was washed with acid and alkali as described by Peterson and Sober (1962). The fly TPI (6,400 units) was loaded onto a DEAE cellulose column (12 x 2.4 cm), which was washed with the elution buffer: 5 mM Tris, 2 mM EDTA, pH 7.8. TPI was eluted from the column by 0.4 M ammonium sulfate in the same buffer; elution rate was 1.9

ml per 5 min.

4. G-100 chromatography: The eluted enzyme solution from the DEAE cellulose column was reconcentrated by Aquacide I (Calbiochem) to a final volume of 5 ml, which was applied onto a Sephadex G-100 column (102 x 14 cm) equilibrated with the elution buffer: 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.8.

The final specific activity of the housefly TPI was 2,412 units/mg (Table 1), comparable to that of the commercially available rabbit TPI crystals (2,000 units/mg). The elution profile by Sephadex G-100 showed that there might be aggregation of the housefly TPI prior to the activity peak (Fig. 1).

Purification of Mosquito Triosephosphate Isomerase

All the steps were carried out at 0-4 C, and Tris-EDTA buffers were used throughout the purification. The enzyme activity was assayed with G-3-P as substrate.

1. Extraction: One gram of mosquito thoraces was homogenized for 1 min in a Sorval omni-mixer with 14 ml of 0.3 M sucrose, 10 mM EDTA, 0.1 M Tris (pH 7.8), and centrifuged at 10,000 x g for 50 min. The supernatant was filtered through cheese cloth; the residue re-homogenized with 4 ml of the extraction buffer, and centrifuged again, and the supernatants from the first and second centrifugation pooled together.

2. Acid precipitation: The pH of the combined supernatants was

adjusted to pH 5.2 by 3 N acetic acid. The precipitate was discarded after centrifuging at 10,000 x g for 30 min. The pH of the supernatant was adjusted back to 7.8 by 2 N NaOH.

3. Buffer exchange by Sephadex G-50: Sephadex G-50 was used as recommended by Knowles and Pon (1968). The G-50 column with a bed volume of 41 ml (1.2 x 35 cm) was equilibrated with 5 mM Tris, 2 mM EDTA (pH 7.8). After the application of the supernatant, the enzyme was eluted with the same buffer with a flow rate of 1 ml/5 min. The eluted volume was reconcentrated to 15 ml by Aquacide I. The increase in the total activity at this step was probably due to the removal of inhibitors (Table 2).

4. DEAE cellulose chromatography: The DEAE cellulose was prepared by the method of Peterson and Sober (1962). The mosquito TPI (8,260 units) was loaded onto a DEAE cellulose column (17 x 2.4 cm), which was equilibrated with the elution buffer: 5 mM Tris, 2 mM EDTA, pH 7.8. The elution rate was 2 ml/4 min. TPI came out with the first protein peak (Fig. 2A).

5. G-100 chromatography: The eluted enzyme solution from the DEAE cellulose chromatography was reconcentrated by Aquacide I to 14 ml. Two 7-ml portions of the enzyme solution were passed through a Sephadex G-100 column (100 x 14 cm), which was equilibrated with the elution buffer: 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.8. There might also be aggregation of mosquito TPI prior to the activity peak (Fig. 2B).

Table 1

Purification of triosephosphate isomerase from the thoraces of the housefly, *M. domestica*. Substrate was 1.2 mM G-3-P.

Fraction	Vol. (ml)	Specific activity (units/mg)	Total activity	Purifi- cation	Yield (%)
1. Crude extract	20	120	23,280	-	100
2. (NH ₄) ₂ SO ₄ step	20	160	13,440	1.3	58
3. DEAE cellulose	9.5	490	4,180	4.1	18
4. Sephadex G-100	34	2,412	4,964	21	21

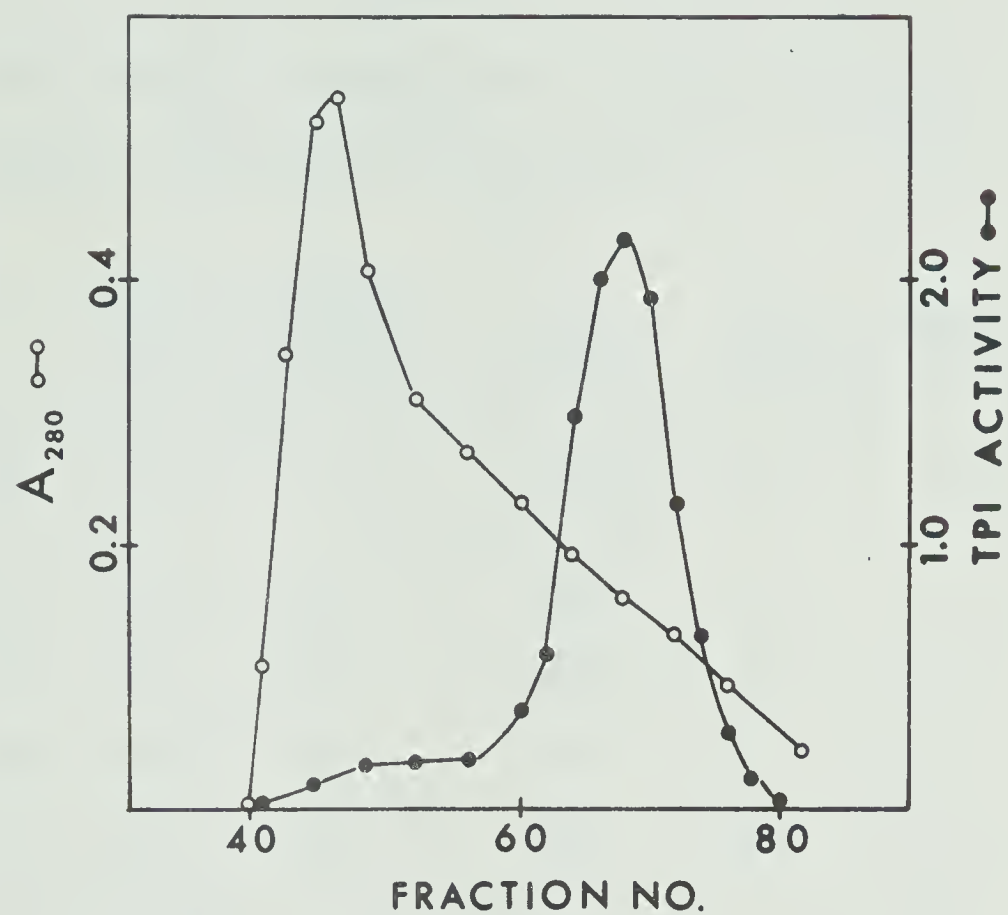


Fig. 1. Elution profile of housefly TPI from Sephadex G-100. 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.8.

Table 2

Purification of triosephosphate isomerase from the mosquito, *A. aegypti*. Substrate was 1.2 mM G-3-P.

Steps	Vol. (ml)	Specific activity (units/mg)	Total activity	Purifi- cation	Yield (%)
1. Crude extract	13.5	77	6,237	-	100
2. Acetic acid precipitation	13.5	160	5,400	2	87
3. Sephadex G-50	35	327	8,260	4	132
4. DEAE cellulose	83	720	7,304	9	117
5. Sephadex G-100	84	2,000	5,376	26	86

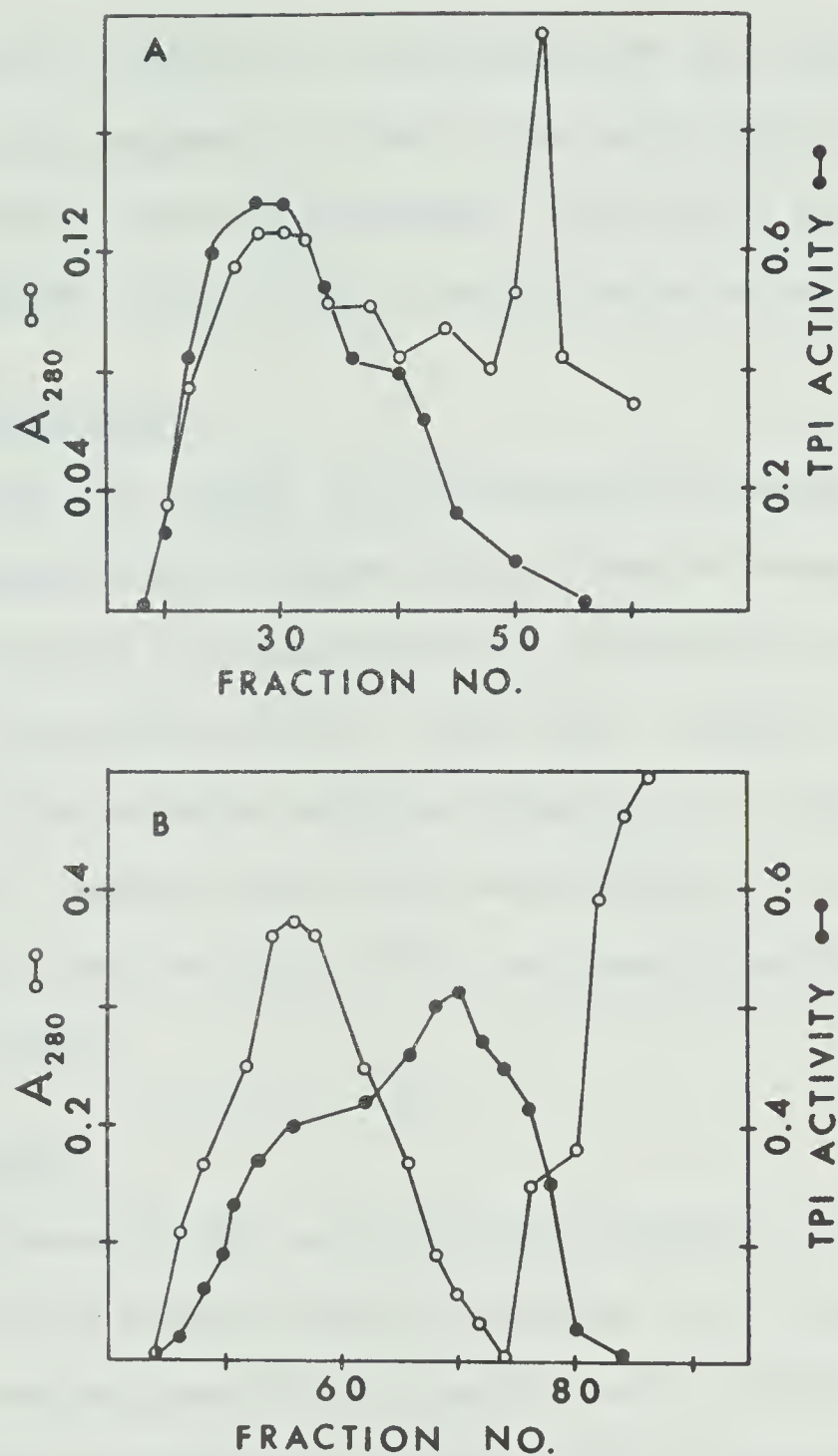


Fig. 2. Elution profiles of mosquito TPI. A. DEAE cellulose; 5 mM Tris, 2 mM EDTA, pH 7.8. B. Sephadex G-100; 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.8.

The final specific activity of the mosquito TPI was 2,000 units/mg (Table 2), also comparable to that of the rabbit TPI crystals. The enzyme was remarkably stable in 50 mM Tris, 5 mM EDTA at 4 C, and its activity remained constant over a 9-month period or more.

Molecular Weight Determination

Molecular weight for the TPI from the mosquito, housefly, and rabbit was determined with a Sephadex G-100 column (Andrews, 1964), which was calibrated with gamma-globulin, haemoglobin, cytochrome C, and myoglobin in the elution buffer: 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.8. The estimated molecular weight for the native TPI from the mosquito, housefly, and rabbit was 60,000 (Fig. 3). This agrees with that found for rabbit TPI by ultracentrifugation (Johnson and Waley, 1967).

Electrophoretic Mobility

The purified housefly TPI, and rabbit TPI were used. The purified housefly TPI was reconcentrated by Aquacide I to 5.5 mg/ml. About 20 λ of the reconcentrated TPI were applied onto a cellulose acetate strip. Both the housefly TPI and the rabbit TPI migrated toward the cathode, faster at pH 8.0 than at pH 6.5 (Fig. 4). But the rabbit TPI was more cationic than the housefly TPI. The enzymic activity corresponded with the stained bands. Burton and Waley (1966) reported that rabbit TPI migrated as a cation. The appearance of a single band, instead of bands, was probably due to the lower resolving power of cellulose acetate.

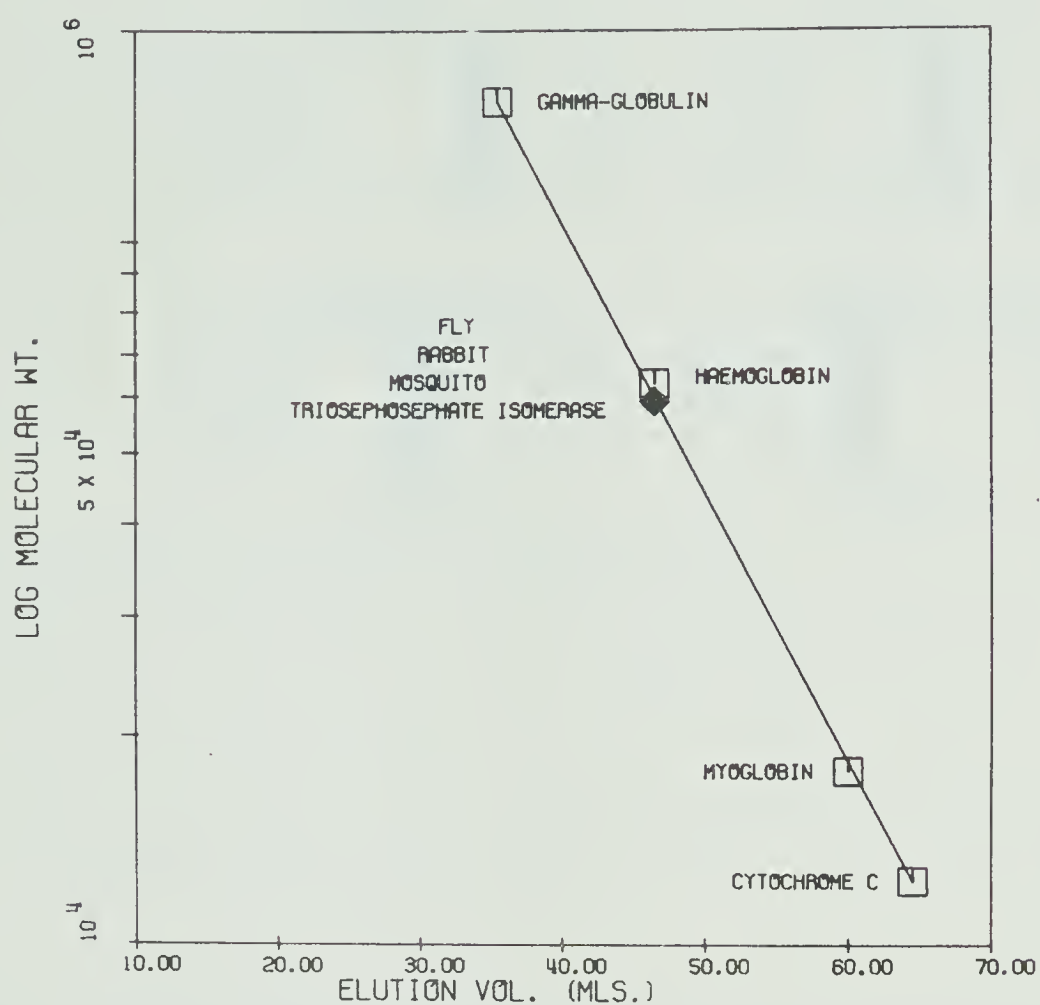


Fig. 3. Estimation of the molecular weight of TPI from the housefly, rabbit and mosquito by Sephadex G-100 according to the method of Andrews (1964). The molecular weight was estimated as 60,000. Elution buffer consisted of 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.8.

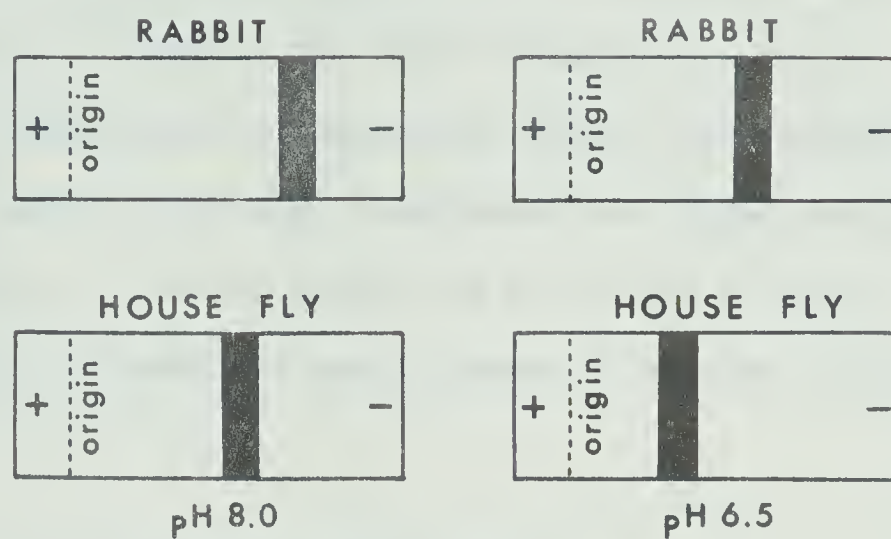


Fig. 4. Electrophoresis of housefly TPI, and rabbit TPI on cellulose acetate at pH 8.0 and pH 6.5. Strip length 12 in; 6 mA, 300 V for 5 hr at 4 C. Stained by Ponceau S.

Kinetic Properties of Mosquito TPI

The kinetic properties of mosquito TPI were studied mainly with DHAP as substrate. Since it was found during the course of my work that most preparations of commercial rabbit α -glycerophosphate dehydrogenase also had TPI activity, experiments with G-3-P were not done for mosquito TPI. I did try to get rid of the TPI activity from the rabbit α -glycerophosphate dehydrogenase by various methods, but to no avail.

The effect of pH (modified assay method)

The mosquito TPI activity was skewed toward the alkaline range; the highest activity occurred at pH 8.8 (Fig. 5). The skewness toward the alkaline range was also observed for the housefly TPI (Fig. 41), and the rabbit TPI (Hartman, 1968; Rieder and Rose, 1959).

Conversion of DHAP to G-3-P at varying amounts of TPI (modified assay method)

The reaction was linear with increasing amounts of mosquito TPI up to about 300 ng (Fig. 6).

The effect of temperature (modified assay method)

The TPI activity was linear between 25 and 40 C (Fig. 7). From 25 to 40 C, the activation energy was 9.94 kcal/mole, and the Q_{10} was 1.7.

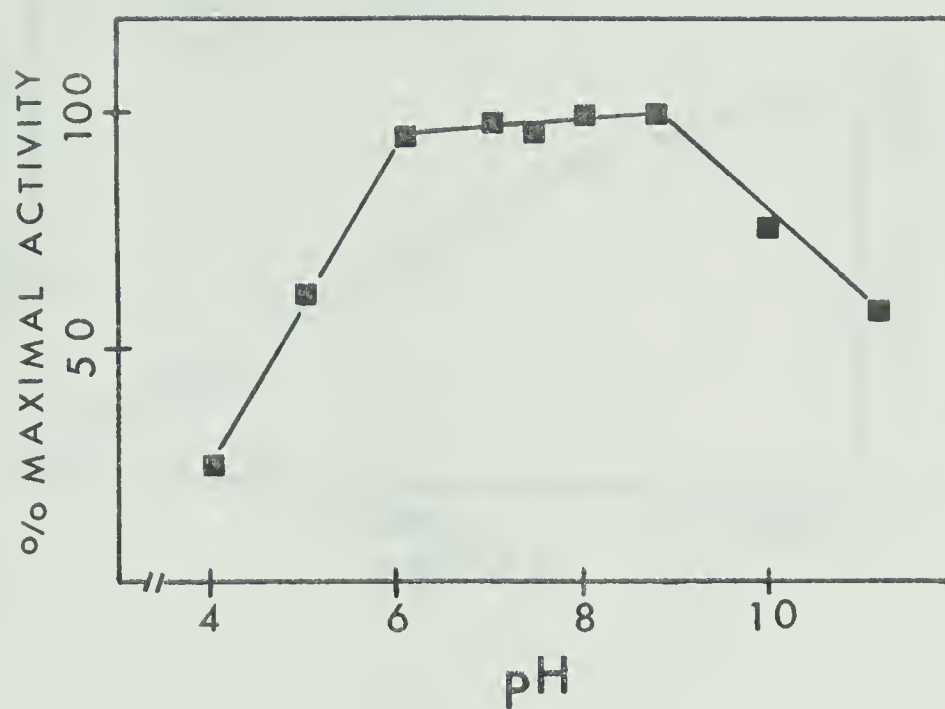


Fig. 5. Effect of pH on the conversion of DHAP (8.96 mM) to G-3-P by mosquito TPI. Temp. 30 C, modified assay method.

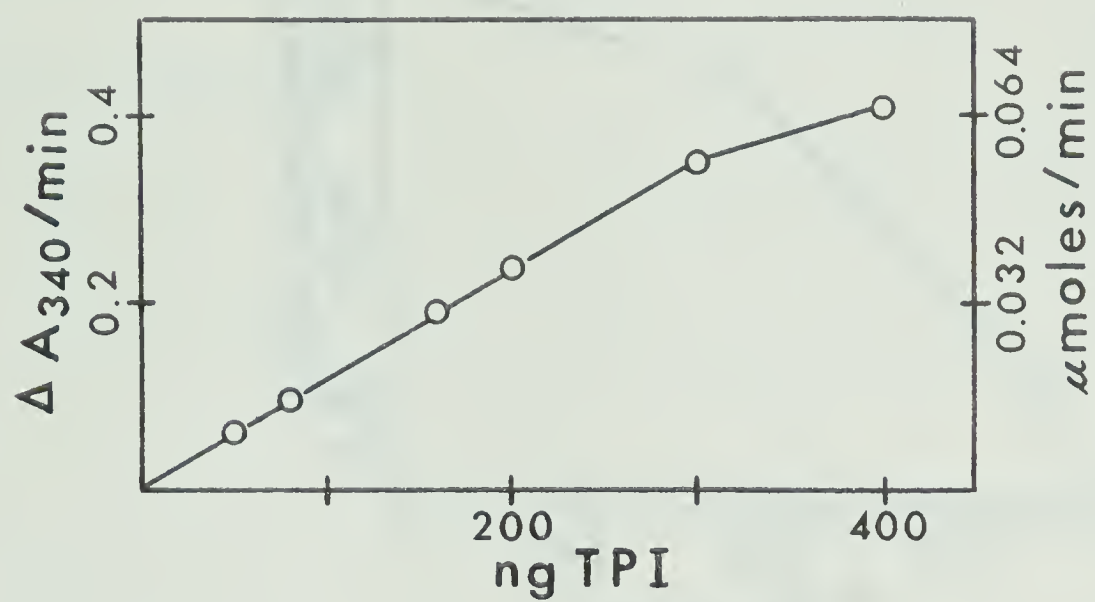


Fig. 6. Conversion of DHAP (6.2 mM) to G-3-P at varying amounts of mosquito TPI. Temp. 30, pH 8.0, modified assay method.

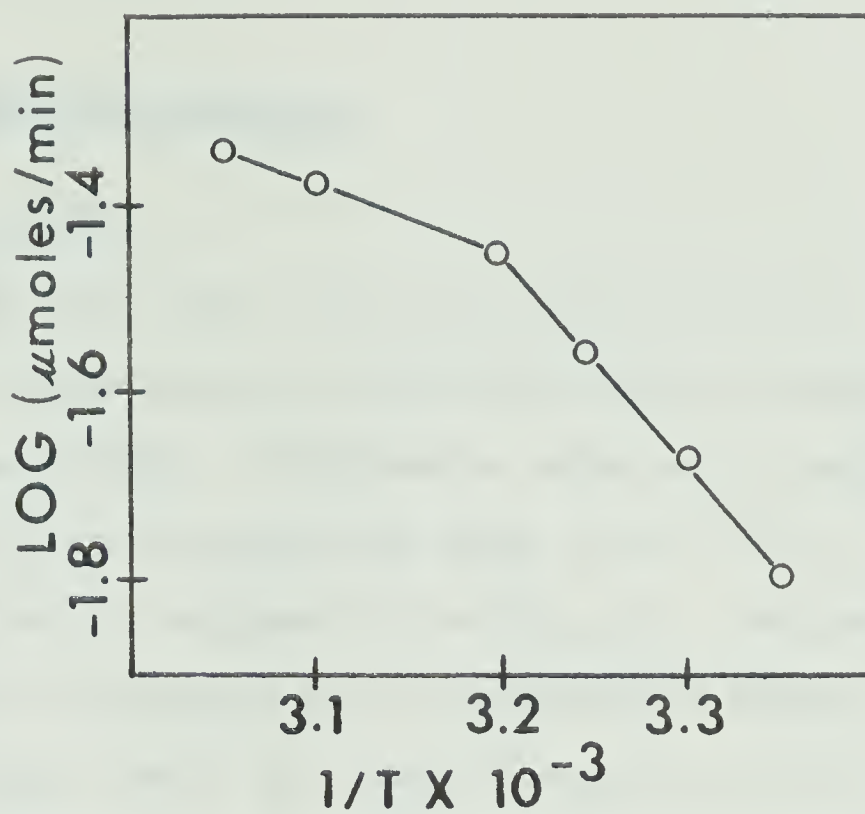


Fig. 7. Effect of temperature on the conversion of DHAP (8.96 mM) to G-3-P by mosquito TPI, pH 8.0. Modified assay method.

The effect of DHAP concentrations

Coupling enzyme method

The reaction was linear over a 7-min period (Fig. 8). The velocity vs. substrate concentration curve will be called the Michaelis curve, as used by Atkinson (1966). The Michaelis curves were sigmoidal when assayed with 40 or 80 ng of mosquito TPI (Figs. 9, 10). This sigmoidicity was borne out by a Wilkinson's plot (Fig. 11). The concave upward phenomenon shown by Wilkinson's plot is indicative of sigmoidicity (Gardiner and Ottaway, 1969). The classical Michaelis-Menton equation was not applicable for the following reasons: (1) The V_m estimated was too high, 2 to 4 times greater than observed (Table 3). The V_m estimated for the experiment with 40 ng TPI was almost the same as that estimated for the determination done with 80 ng TPI. (2) The K_m was too large, and did not seem to agree with the Michaelis curves (Table 3). (3) The Wilkinson's plots were non-linear (Fig. 11). This non-linearity was repeatable, and was probably not due to experimental errors because K_m determinations with G-3-P as substrate, and within the same range of A_{340} readings, did not show any deviations (Figs. 42, 45). Gardiner and Ottaway (1969) have discussed the merits of Wilkinson's plot in detecting sigmoidicity or departure from the simple Michaelis-Menton equation. The statistical errors are less when Wilkinson's plot is used (Wilkinson, 1961).

Subsequently, it was found that the reaction catalyzed by TPI with DHAP as substrate could be approximated by plotting S^2/V against S^2 , giving $V_m(s)$ close to the observed (Figs. 9B, 10B; Table 3).

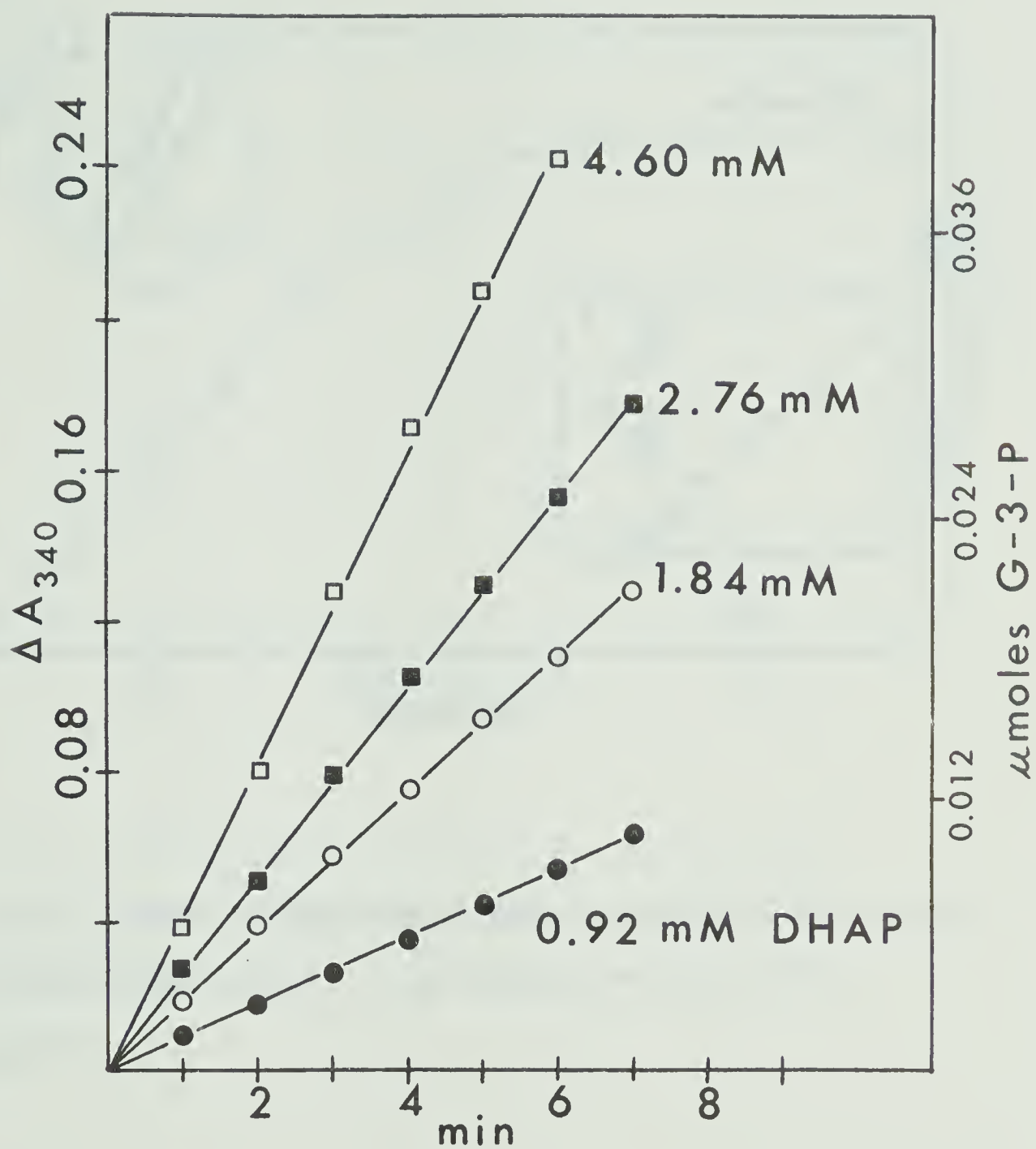


Fig. 8. Conversion of DHAP to G-3-P by mosquito TPI vs. time by the coupling enzyme method. Temp. 30 C, pH 8.0, 40 ng mosquito TPI.

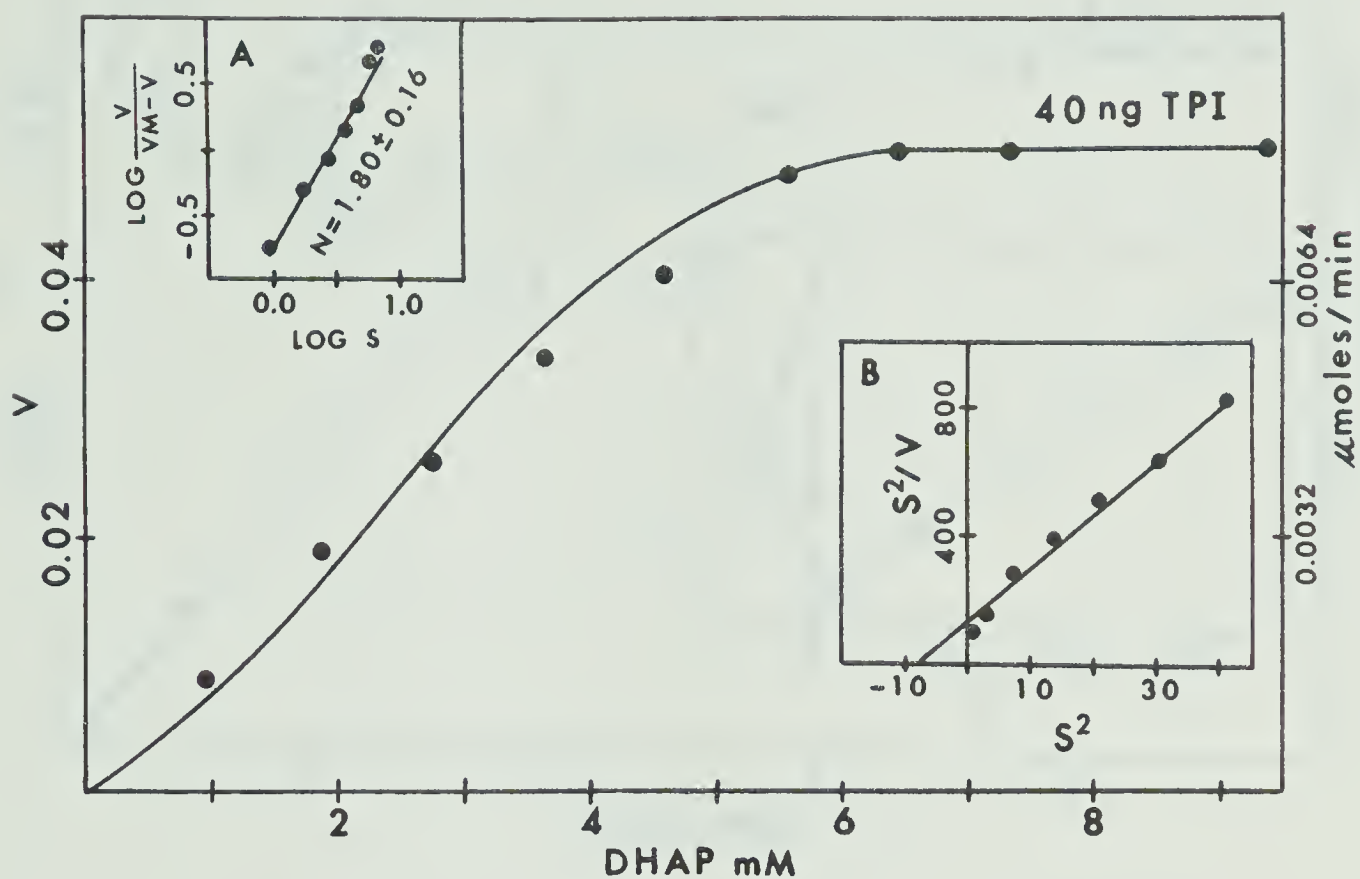


Fig. 9. Effect of DHAP concentrations on mosquito TPI activity by the coupling enzyme method. 40 ng mosquito TPI, temp. 30 C.

$V = \Delta A_{340}/\text{min.}$

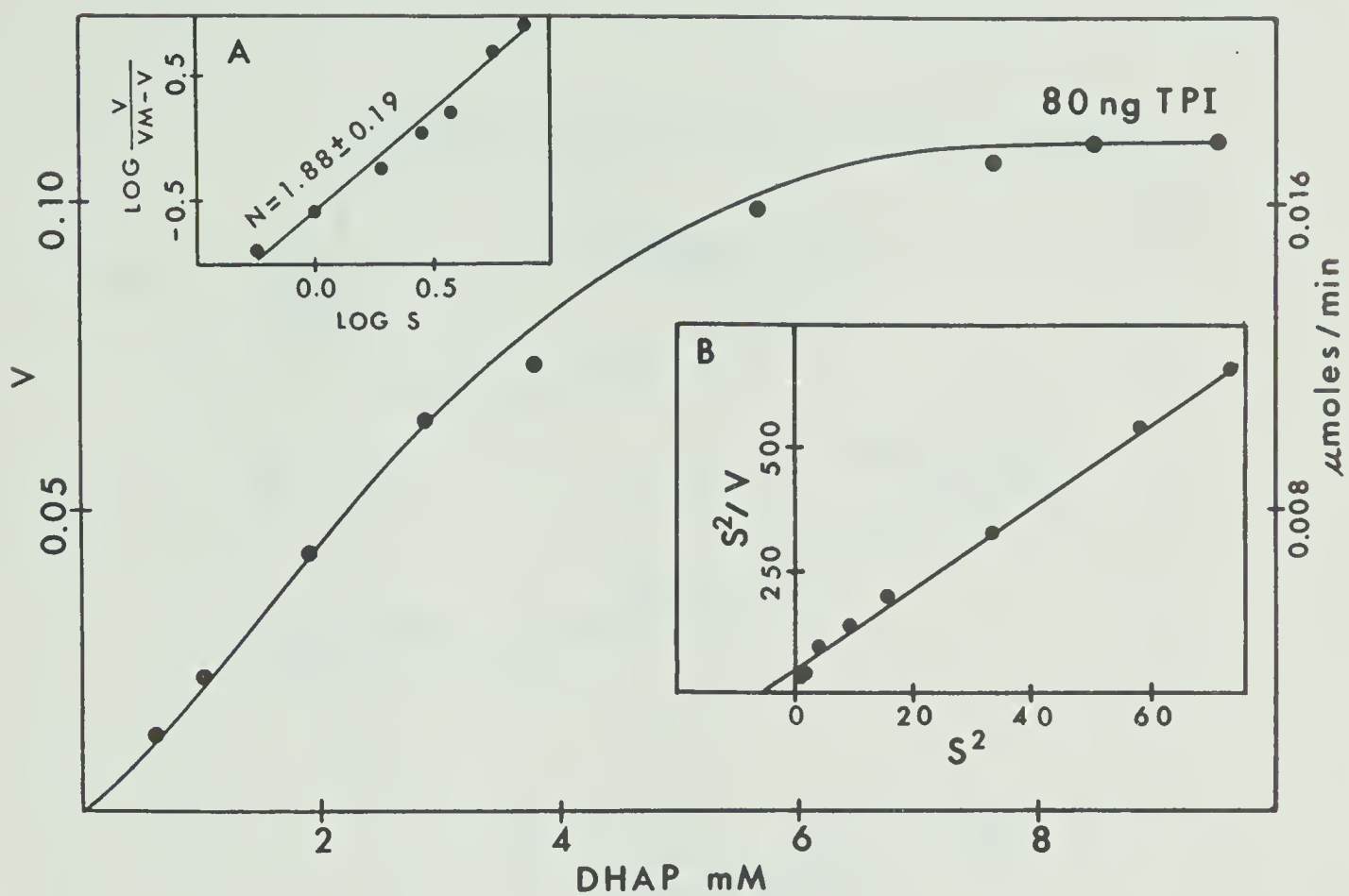


Fig. 10. Effect of DHAP concentrations on mosquito TPI activity by the coupling enzyme method. 80 ng mosquito TPI, temp. 30 C. $V = \Delta A_{340}/\text{min}$.

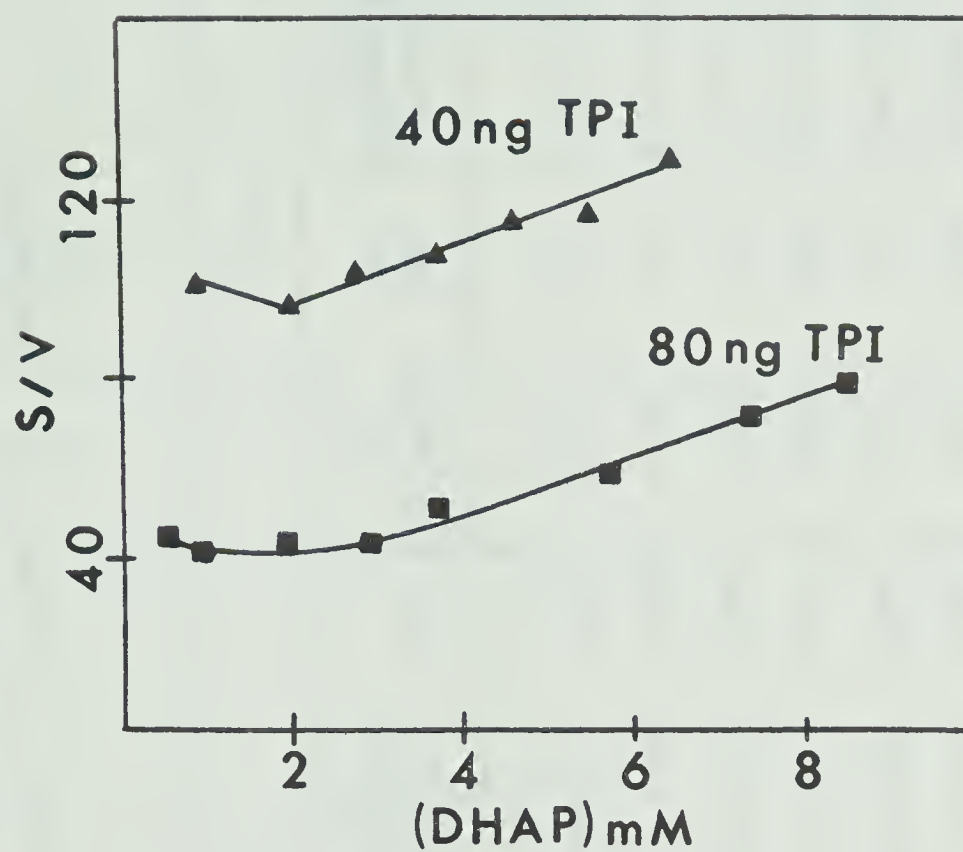


Fig. 11. Wilkinson's plot of kinetic data from Figs. 9 and 10 according to the equation: $S/V = K_m/V_m + S/K_m$.

Table 3

Summary of the estimation of kinetic parameters for mosquito TPI by the coupling enzyme method, modified assay method, and pseudo first order method.

Figs. 9, 10, 15, 16.

Experiment Exptl. V _m (μmoles/min)	S ² /V vs. S ²				S/V vs. S				
	V _m estimated	Apparent K _m (mM)	95% Confidence limits	R [†]	V _m estimated	K _m (mM)	95% Confidence limits	R	
40 ng*	0.008	0.009	2.75	1.95-3.52	0.992	0.034	19.07	11.66-40.13	0.922
80 ng*	0.017	0.019	2.33	1.71-2.87	0.998	0.036	8.19	5.67-12.33	0.970
1 min assay	0.024	0.024	2.23	0.86-3.20	0.994	0.039	5.15	3.20-8.53	0.981
2 min	0.021	0.022	2.02	1.11-2.72	0.997	0.036	5.53	3.53-8.96	0.981
3 min	0.020	0.021	2.13	1.05-2.94	0.997	0.034	5.51	4.86-6.27	0.995
4 min	0.017	0.020	2.16	1.07-2.97	0.996	0.031	5.79	4.26-8.02	0.991
Pseudo 1st order method	0.021	0.022	1.86	0.97-2.51	0.998	0.032	3.93	2.79-5.50	0.992

* Coupling enzyme method

^+R = Coefficient of correlation

This apparent fit of the data, by plotting S^2/V against S^2 , suggests that the reaction can be approximated by the following equation:

$$S^2/V = K/V_m + S^2/V_m \quad (10)$$

which is actually the Hill equation in the Michaelis-Menton form:

$$V = \frac{V_m S^2}{K + S^2} \quad (11)$$

where K has a different meaning from the Michaelis-Menton constant, K_m (Atkinson, 1966). For equation 10, when S^2/V equals zero, $-K$ is equal to S^2 ; therefore, the apparent K_m or $S_{0.5}$ can be determined from the square root of S^2 . The apparent K_m or $S_{0.5}$ is strictly defined as "half-saturating concentration of substrate" (Atkinson, 1966). With 40 ng mosquito TPI, the apparent K_m was 2.75 mM; confidence limits from 1.95-3.52 (Table 3). The apparent K_m was 2.33 mM for the experiment done with 80 ng TPI; confidence limits from 1.71-2.87 mM (Table 3). Judging from the Michaelis curves, the apparent $K_m(s)$ estimated fitted quite well the kinetic behavior of mosquito TPI regarding DHAP as substrate.

The V_m estimated from equation 10 was used to calculate the Hill coefficient, N , in the Hill plot. The N values were about 1.8 (Figs. 9A, 10A). Atkinson, Hathaway and Smith (1965) estimated the V_m for yeast isocitrate dehydrogenase by reciprocal plots of rate against the 4th power of substrate concentrations. However, there can be fallacies when interpreting the Hill plots since the V_m estimated assumed a second order reaction already. Using the experimental V_m , N was 2.27 ± 0.46 at 40 ng TPI, and 1.66 ± 0.11

at 80 ng TPI.

Assuming equation 11 is applicable for approximating the kinetic behavior of TPI with DHAP as substrate, differentiation of this equation gives:

$$\frac{dV}{dS} = \frac{2 V_m K S}{(K + S^2)^2} \quad (12)$$

If $dV/dS = 0$, $S = 0$, or $S = \infty$. The second differential of eq. 11 is given by:

$$\frac{d^2V}{dS^2} = \frac{2 V_m K^3 - 4 V_m K^2 S^2 - 6 V_m K S^4}{(K + S^2)^4} \quad (13)$$

If $d^2V/dS^2 = 0$

$$2 V_m K^3 - 4 V_m K^2 S^2 - 6 V_m K S^4 = 0$$

$$(K - 3 S^2) (K + S^2) = 0$$

$$S = \pm \sqrt{K/3}, \text{ or } S = \pm \sqrt{-K}$$

Because $S = \pm \sqrt{-K}$ is imaginary, therefore, $S = \pm \sqrt{K/3}$. Since

$S \geq 0$, $S = \sqrt{K/3}$. Substituting $\sqrt{K/3}$ into eq. 11,

$$V = \frac{V_m (K/3)}{K + K/3} \text{ or, } V = V_m/4$$

Using the third derivative test, it can be shown that $(\sqrt{K/3}, V_m/4)$ is a point (and the only point) of inflection for the curve. Hence, the location for the inflection point of equation 11 is governed by the kinetic parameters K , and V_m . Unless appropriate scales are chosen, and experimental errors minimized, the inflection point may not be noticeable.

Modified assay method

Using the modified method, the reaction was curved over a 16-min period (Fig. 12). The effect of DHAP concentrations upon mosquito TPI activity was studied by incubating the enzyme for 1 min, 2 min and 3 min in the assay medium. On the assumption of initial rate kinetics, the Michaelis curves were also slightly sigmoidal (Fig. 13). Wilkinson's plot of the same data showed that the lines for 2-min and 3-min assays were concave (Fig. 14). By plotting S^2/V against S^2 (Fig. 15), the $V_m(s)$ and apparent $K_m(s)$ also fitted the Michaelis curves quite well (Table 3). The apparent K_m was about 2 mM.

Because the reaction catalyzed by TPI is freely reversible, and because DHAP is the favored product, initial reaction rates were also determined by the method of Engers, Bridger and Madsen (1969). The effect of back reaction on the time course of enzymic reactions has been discussed by McGilvery (1970). Pseudo first order reaction rate constants can be obtained by the equation:

$$k t = \ln \frac{A}{A-C} \quad (14)$$

where k is the pseudo first order reaction rate constant, A the concentration of product at equilibrium, and C the amount of product present at time t . The rate constant for each DHAP concentration was determined from 4 assays done at an interval of 1 min up to 4 min, and by plotting t against $\ln(A/A-C)$, k was obtained as the slope. The initial rates were then estimated by multiplying $k(s)$ with the concentrations of G-3-P calculated to be present at equilibrium.

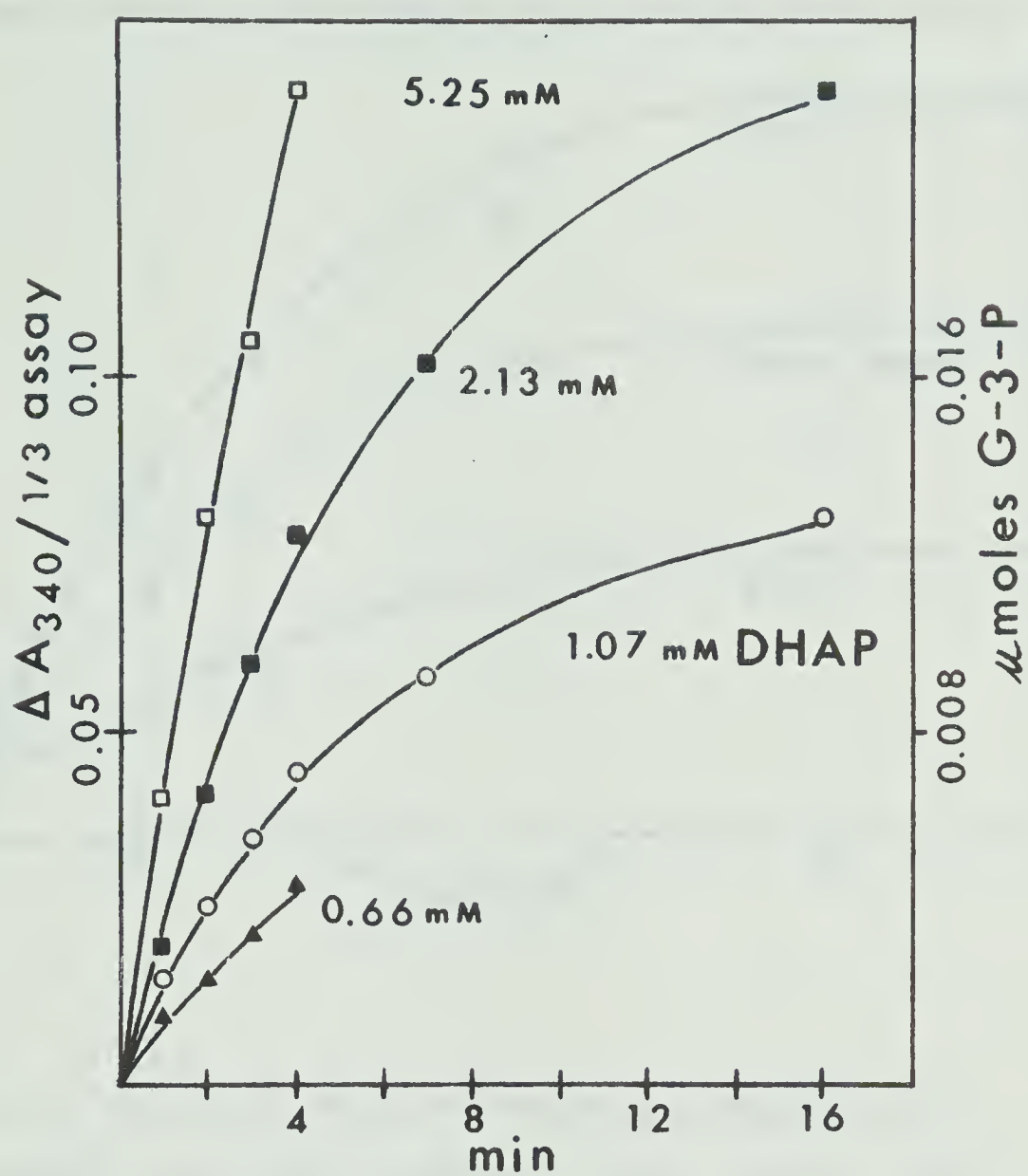


Fig. 12. Conversion of DHAP to G-3-P by mosquito TPI vs. time by the modified assay method. 80 ng mosquito TPI, temp. 30 C, pH 8.0

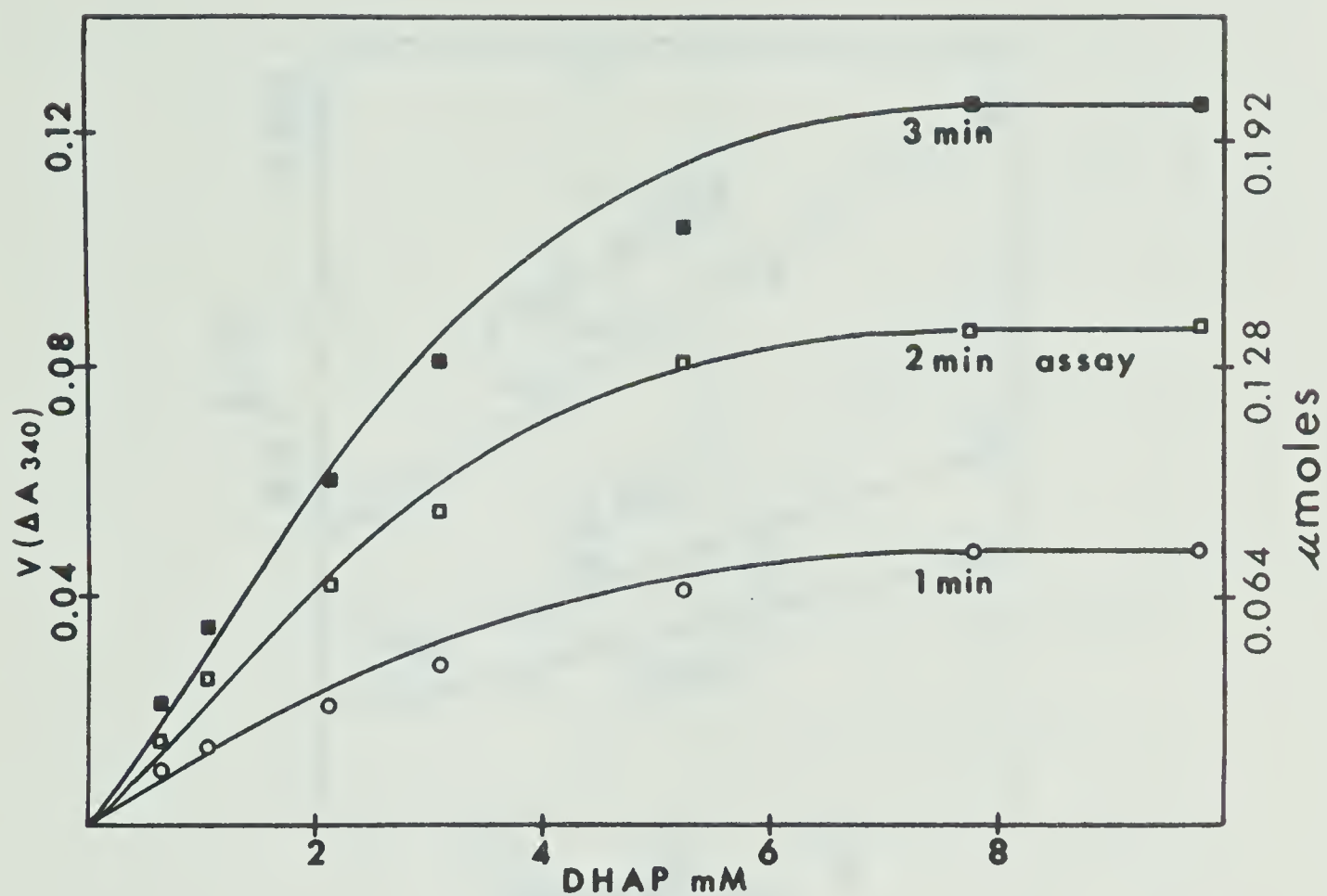


Fig. 13. Effect of DHAP concentrations on mosquito TPI activity assayed by the modified method at 1 min, 2 min and 3 min. 80 ng TPI, temp. 30 C, pH 8.0.

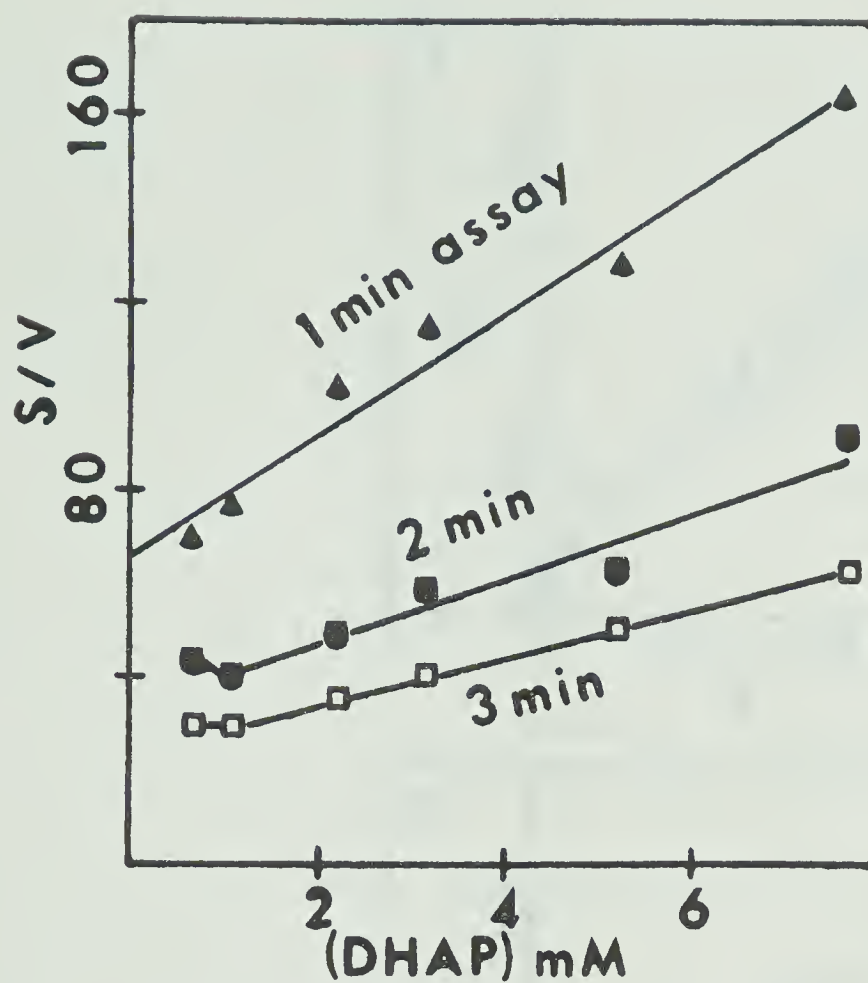


Fig. 14. Wilkinson's plot of kinetic data from Fig. 13 according to the equation: $S/V = K_m/V_m + S/K_m$

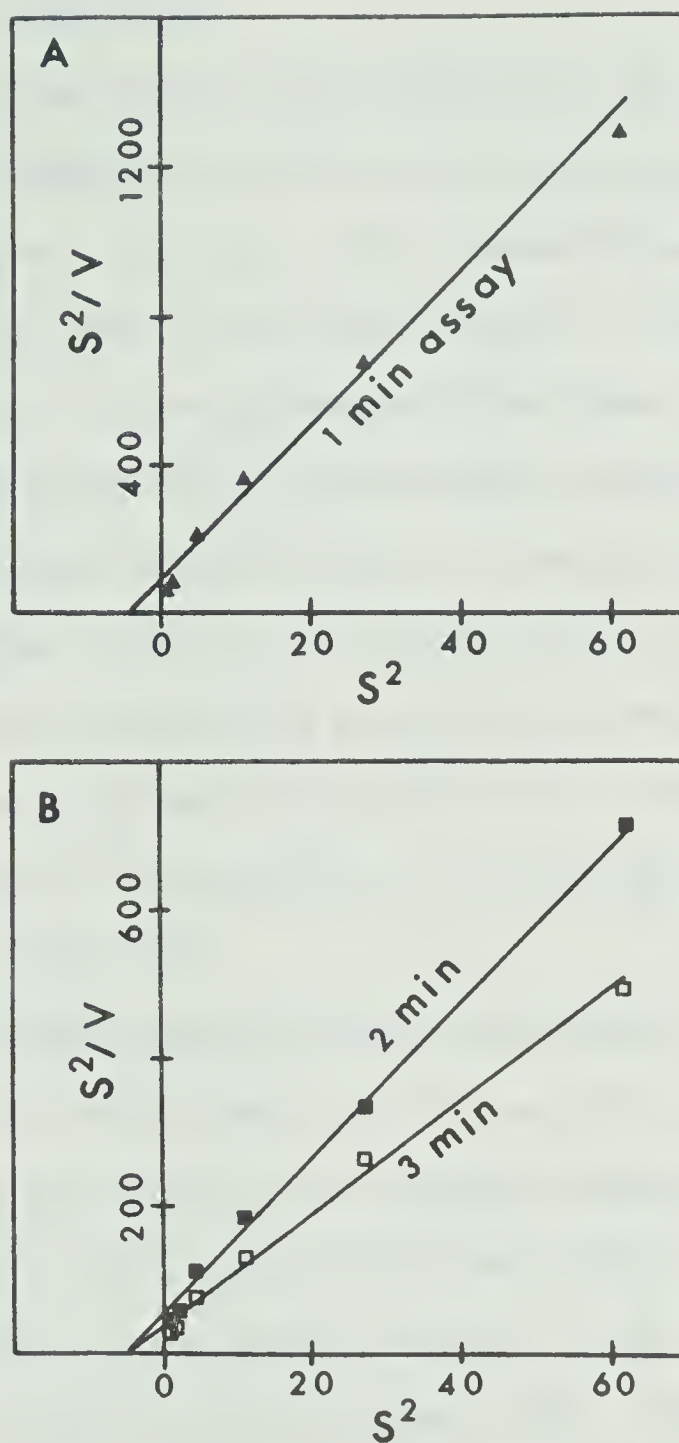


Fig. 15. Wilkinson's plot of kinetic data from Fig. 13 according to the equation: $S^2/V = K/V_m + S^2/V_m$.

The equilibrium constant was taken as 22.

The Michaelis curve of the initial rates obtained by the pseudo first order method was sigmoidal as well, and concavity was observed by plotting S/V against S (Fig. 16). The apparent K_m was 1.86 mM, with confidence limits from 0.97–2.51 mM (Table 3). The V_m estimated was about the same as those estimated by the 1-min, 2-min, and 3-min assays. Because the kinetic parameters estimated for the 2-min and 3-min assays were about the same as those calculated by the pseudo first order method, initial rate kinetics is still applicable when using the rates determined by the 2-min, or 3-min assays. In all the subsequent experiments with mosquito TPI, rates from 2-min, or 3-min were used, and the majority of the experiments were performed with 170 ng mosquito TPI.

Apparent K_m determinations (modified assay method) were also carried out at 25 C, and 40 C; at 30 C again with 170 ng TPI. The apparent $K_m(s)$ did not change significantly with changing temperature, 1.98 mM DHAP at 25 C, 1.88 mM at 30 C, and 2.0 mM at 40 C (Figs. 17, 18, 19, 20; Table 4). The average apparent K_m was 1.95 mM DHAP. The N values were between 1.7 and 1.8 (Fig. 18B, 19B, 20B). Using the experimental V_m , the values for N became 2.06 ± 0.55 at 25 C, 1.57 ± 0.11 at 30 C, and 2.32 ± 0.44 at 40 C.

Inhibitors

All the inhibition experiments were done with the modified assay method.

Most of the inhibitors found exerted mixed inhibition upon

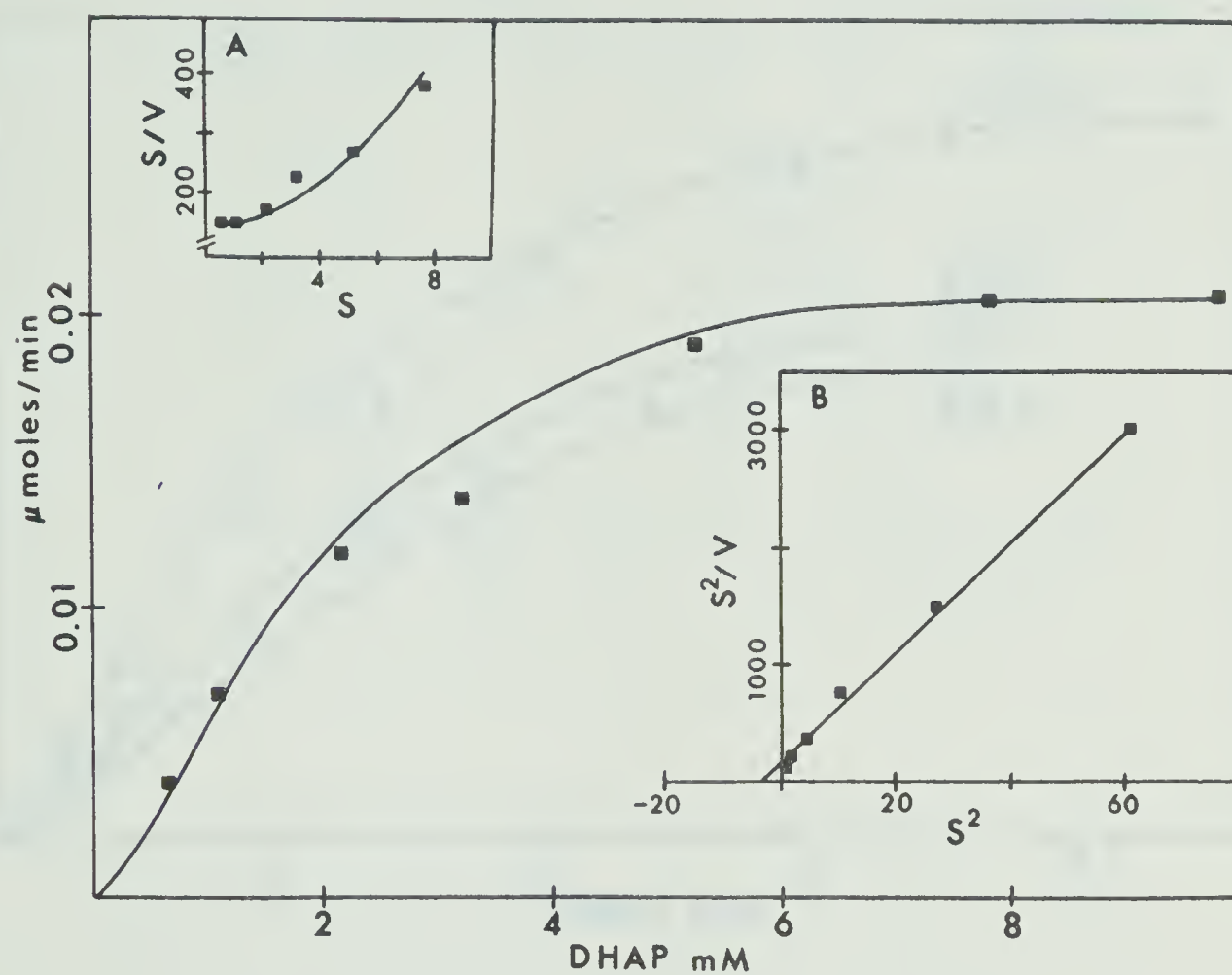


Fig. 16. Michaelis plot of the effect of DHAP concentrations on mosquito TPI activity (modified assay method). The reaction rates were determined according to the procedure of Engers, Bridger and Madsen (1969) on the basis of pseudo first order reaction. 80 ng TPI, temp. 30 C, pH 8.0.

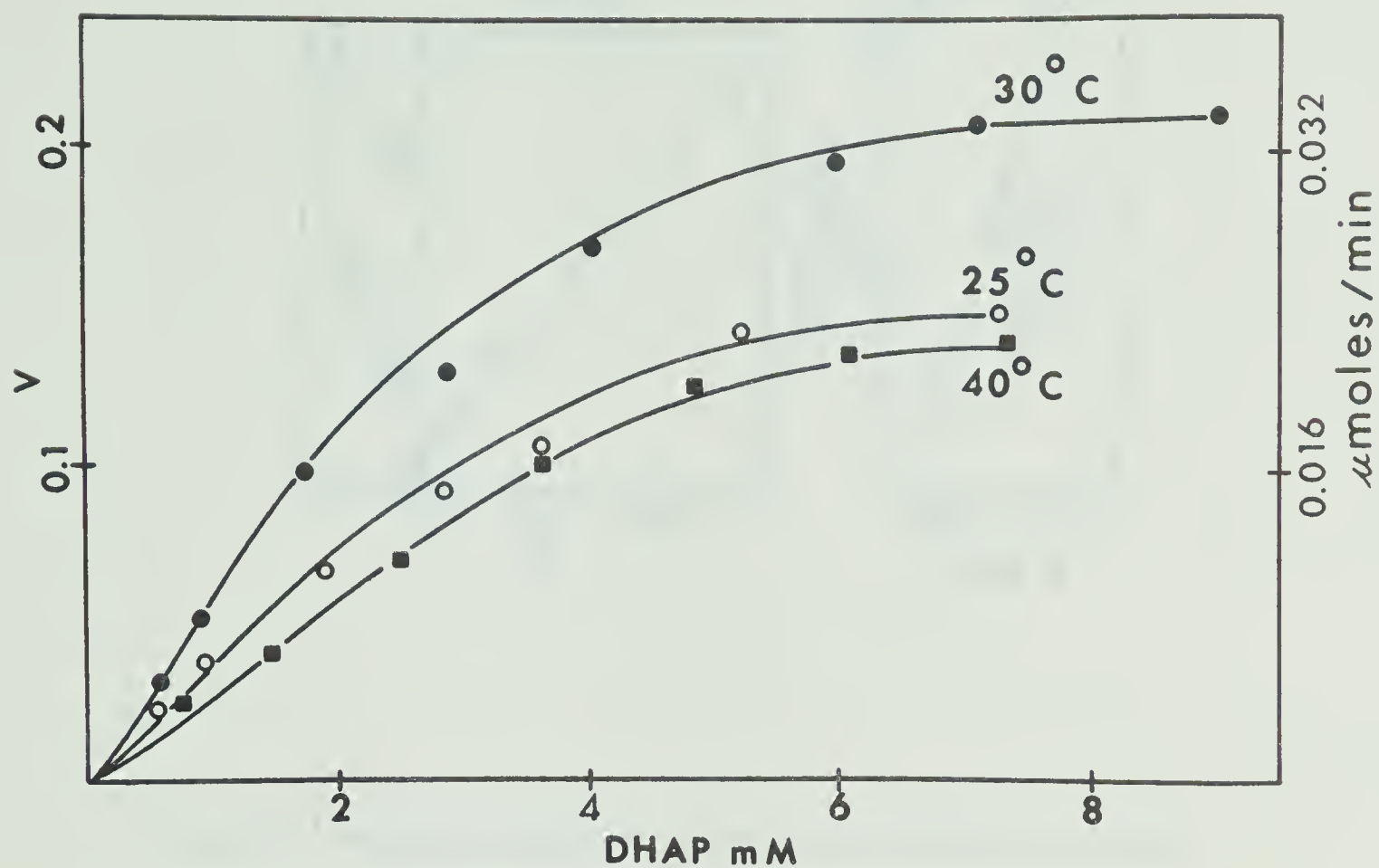


Fig. 17. Effect of DHAP concentrations on mosquito TPI activity at 25 C, 30 C and 40 C. 170 ng mosquito TPI at 25 C and 30 C, 28.3 ng at 40 C; pH 8.0; modified assay method; $V = \Delta A_{340}/\text{min}$.

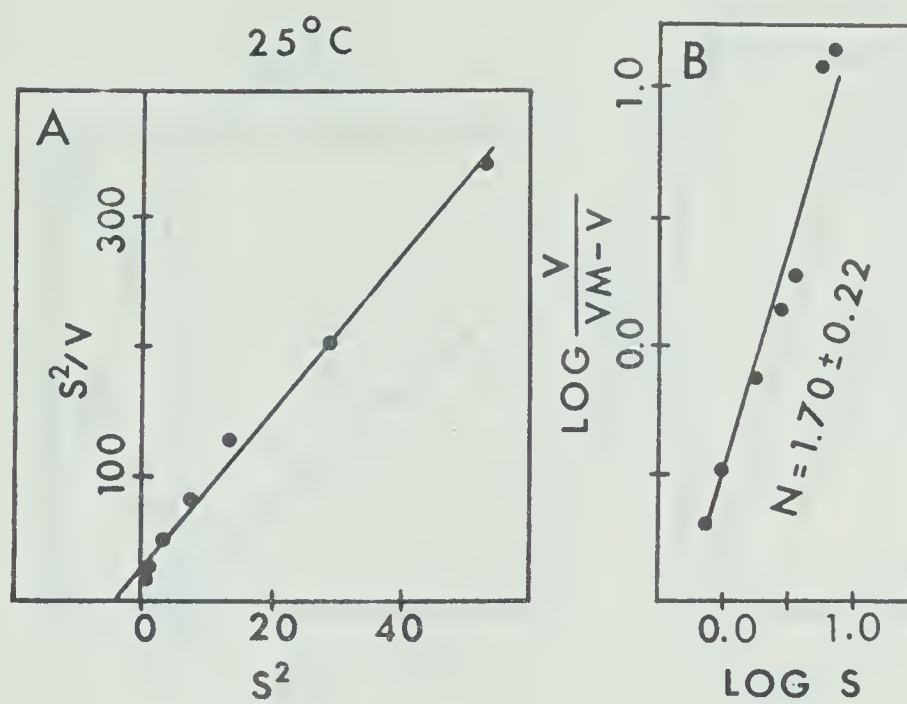


Fig. 18. Modified Wilkinson's plot, and Hill plot of the effect of DHAP concentrations on mosquito TPI activity at 25 C. Data from Fig. 17.

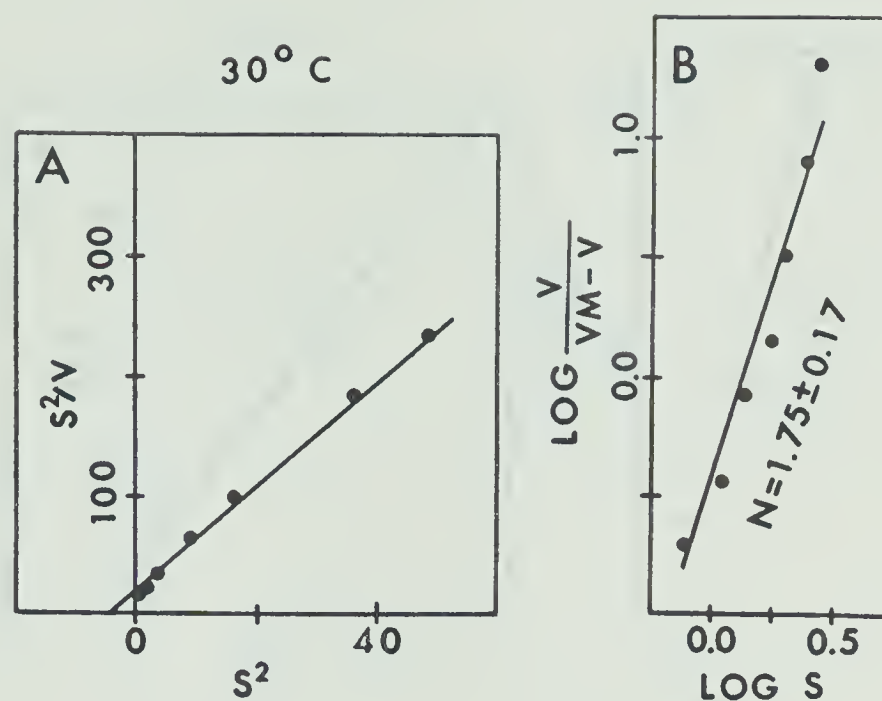


Fig. 19. Modified Wilkinson's plot, and Hill plot of the effect of DHAP concentrations on mosquito TPI activity at 30 C.

Data from Fig. 17.

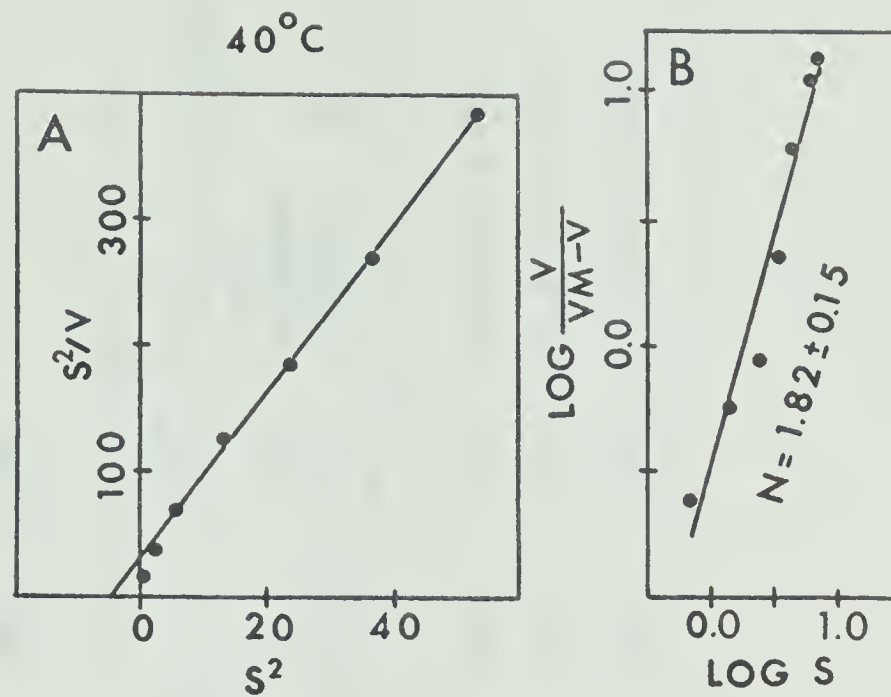


Fig. 20. Modified Wilkinson's plot, and Hill plot of the effect of DHAP concentrations on mosquito TPI activity at 40 C. Data from Fig. 17.

Table 4

Summary of the estimation of kinetic parameters for mosquito TPI at
25 C, 30 C, and 40 C by: S^2/V vs. S^2 ; S/V vs. S . Figs. 18-20.

Temp. C	Exptl. V_m ($\mu\text{moles/min}$)	S^2/V vs. S^2			S/V vs. S		
		V_m estimated	Apparent K_m (mM)	95% Confidence limits	R^*	V_m estimated	K_m (mM) 95% Confidence limits R
25	0.024	0.026	1.98	1.05-2.70	0.995	0.042	5.05 3.56-7.28 0.984
30	0.033	0.035	1.88	1.16-2.45	0.997	0.058	4.81 3.74-6.59 0.990
40	0.022	0.023	2.00	1.44-2.49	0.998	0.040	5.04 3.74-6.84 0.989

*R = Coefficient of correlation

mosquito TPI. By plotting S/V against S , concavity became more conspicuous for some of the experiments with inhibitors (Fig. 21). α -Glycero-P was a mixed inhibitor (Fig. 22). Inorganic phosphate (Pi) was one of the most effective inhibitors for TPI (Fig. 23). The effect of Pi was studied further, and will be presented in a later section. Folic acid was probably the most potent inhibitor (Fig. 24). Because of the interference with transmittance at higher concentrations of folic acid, its effect was not explored any further. Krebs cycle intermediates, citrate, succinate and oxaloacetate were inhibitors (Figs. 24, 25). It was found that ADP and ATP were inhibitors also (Fig. 26). The effects of these inhibitors are summarized in Table 5. All the N values were between 1.7 and 1.9.

Among other carboxylic acids studied, glutamate, glutaric acid, adipic acid, DL-glyceraldehyde, pyruvate, and proline were not inhibitors (Table 6). The nucleotides CTP, CDP, UTP and GTP were also inhibitors; but 5'-AMP and 3',5'-AMP were ineffective (Table 6).

The effects of some inhibitors were studied by way of Dixon plot, which is useful in detecting non-linearity in inhibition studies (Cleland, 1967). The inhibitory pattern of α -glycero-P was non-linear at 3 different concentrations of DHAP (Fig. 27). Hill plot of the same data showed an average N of 1.7 (Fig. 27A). If N were taken as the number of moles of α -glycero-P bound per mole of enzyme, then the observed N , 1.7, agrees well with the findings of Hartman (1968) that 1.7 moles of IAP were bound per mole of rabbit

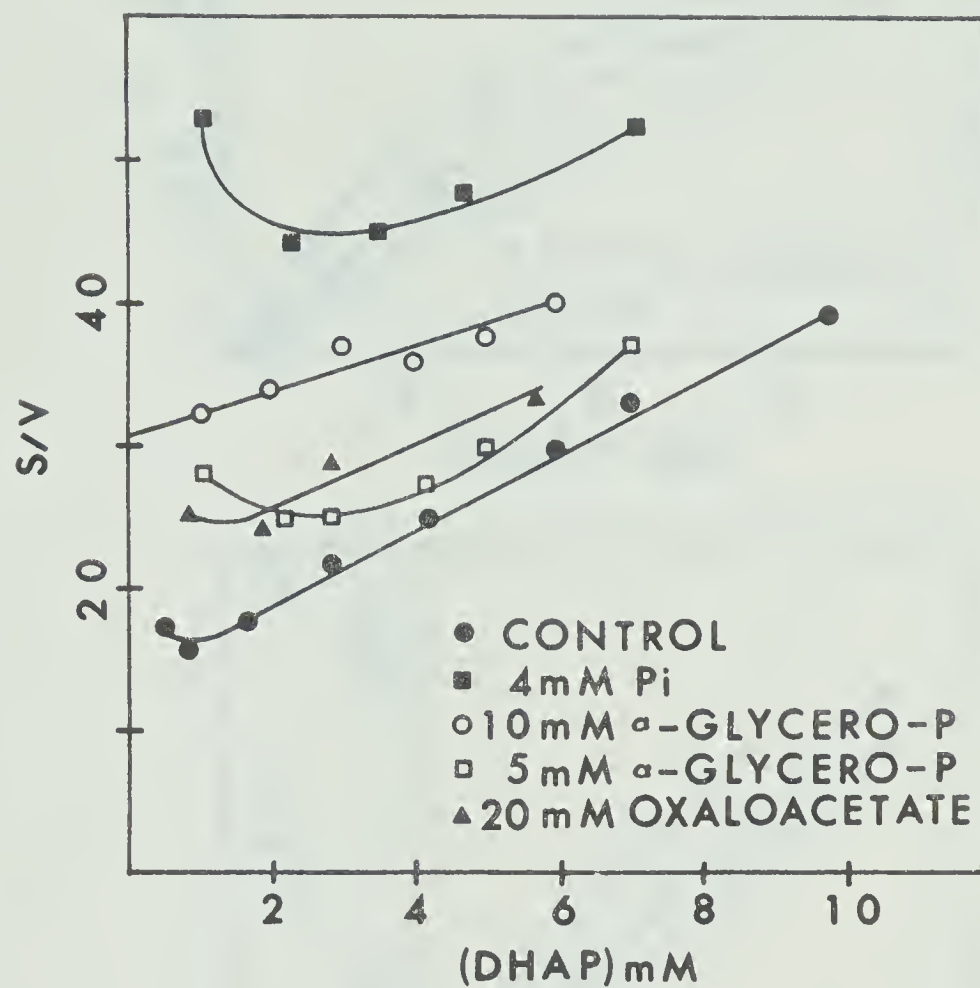


Fig. 21. Wilkinson's plot of kinetic data (DHAP to G-3-P) for mosquito TPI according to the equation: $S/V = K_m/V_m + S/V_m$. The same data also appear in Figs. 19, 22, 23 and 25. 170 ng mosquito TPI, temp. 30 C, modified assay method.

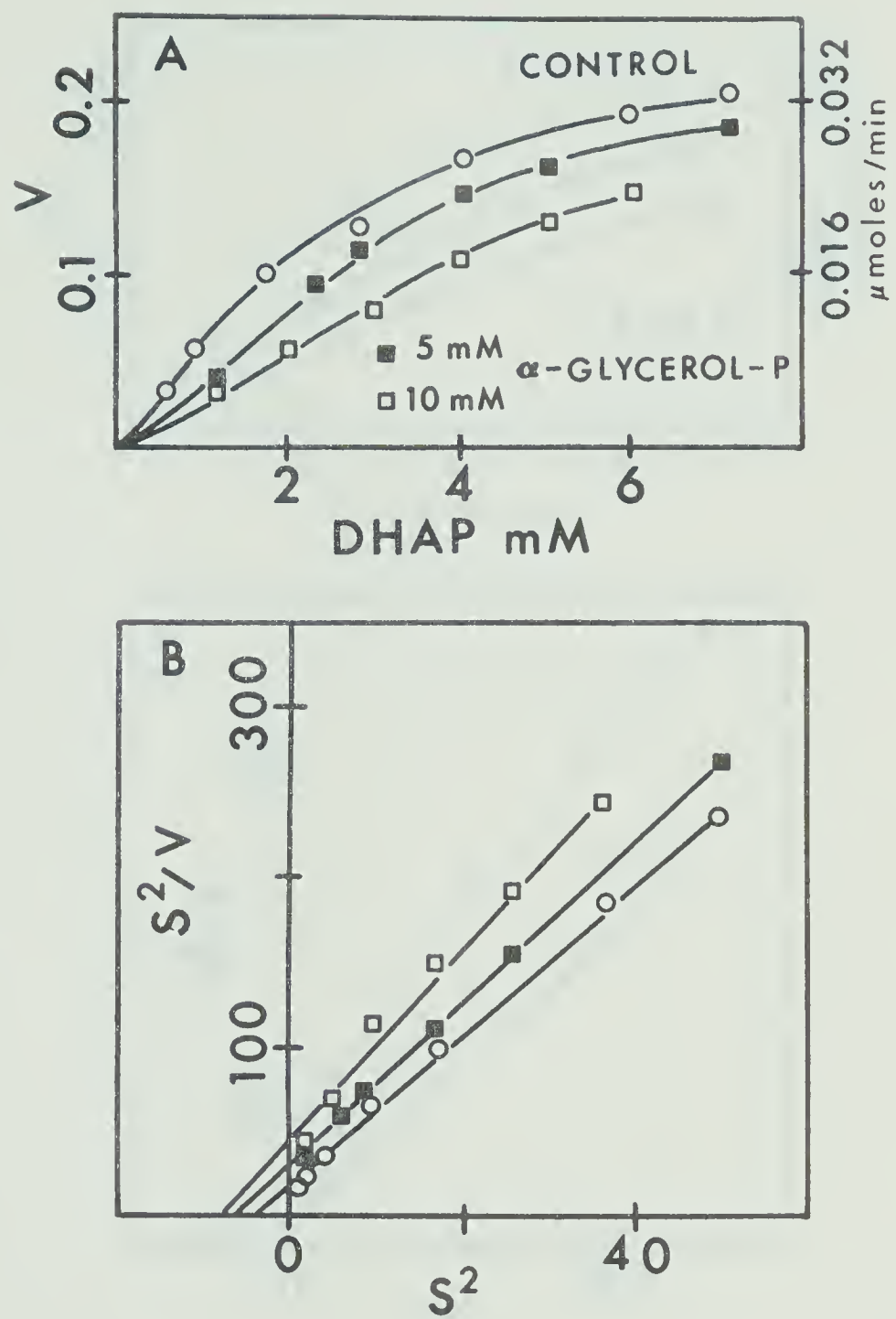


Fig. 22. Effect of DL- α -glycerophosphate on mosquito TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

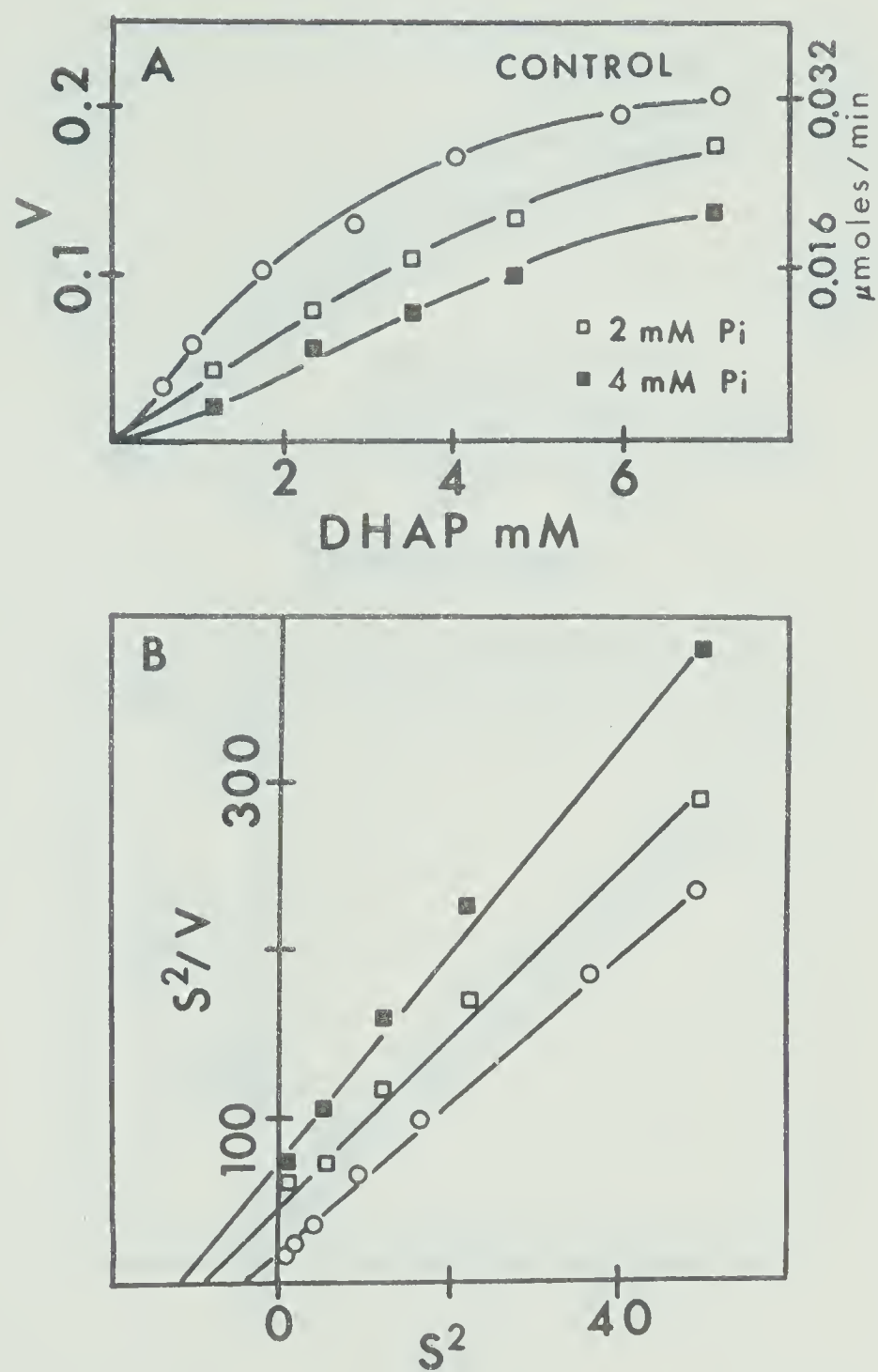


Fig. 23. Effect of Pi on mosquito TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

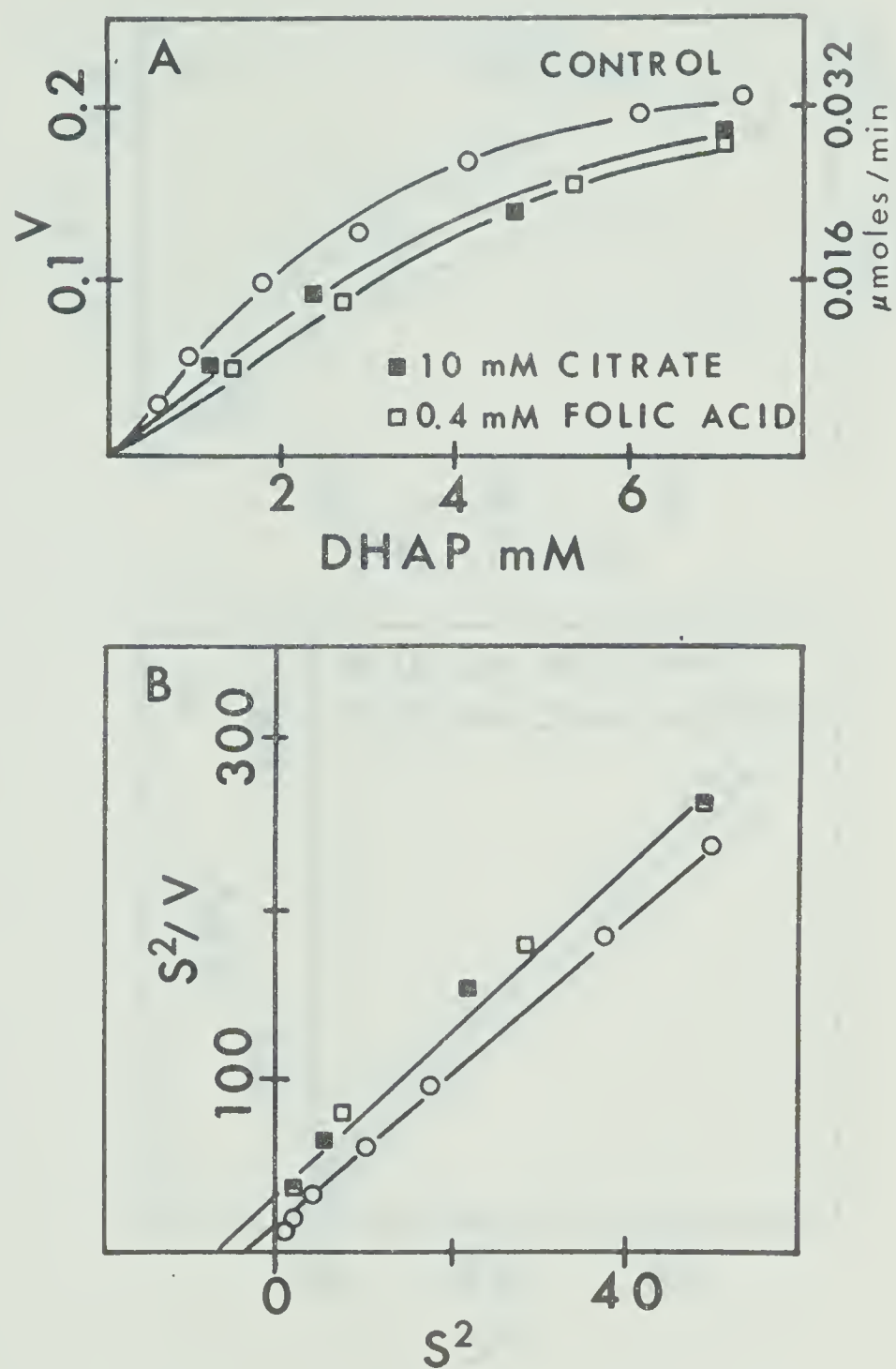


Fig. 24. Effect of citrate and folic acid on mosquito TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

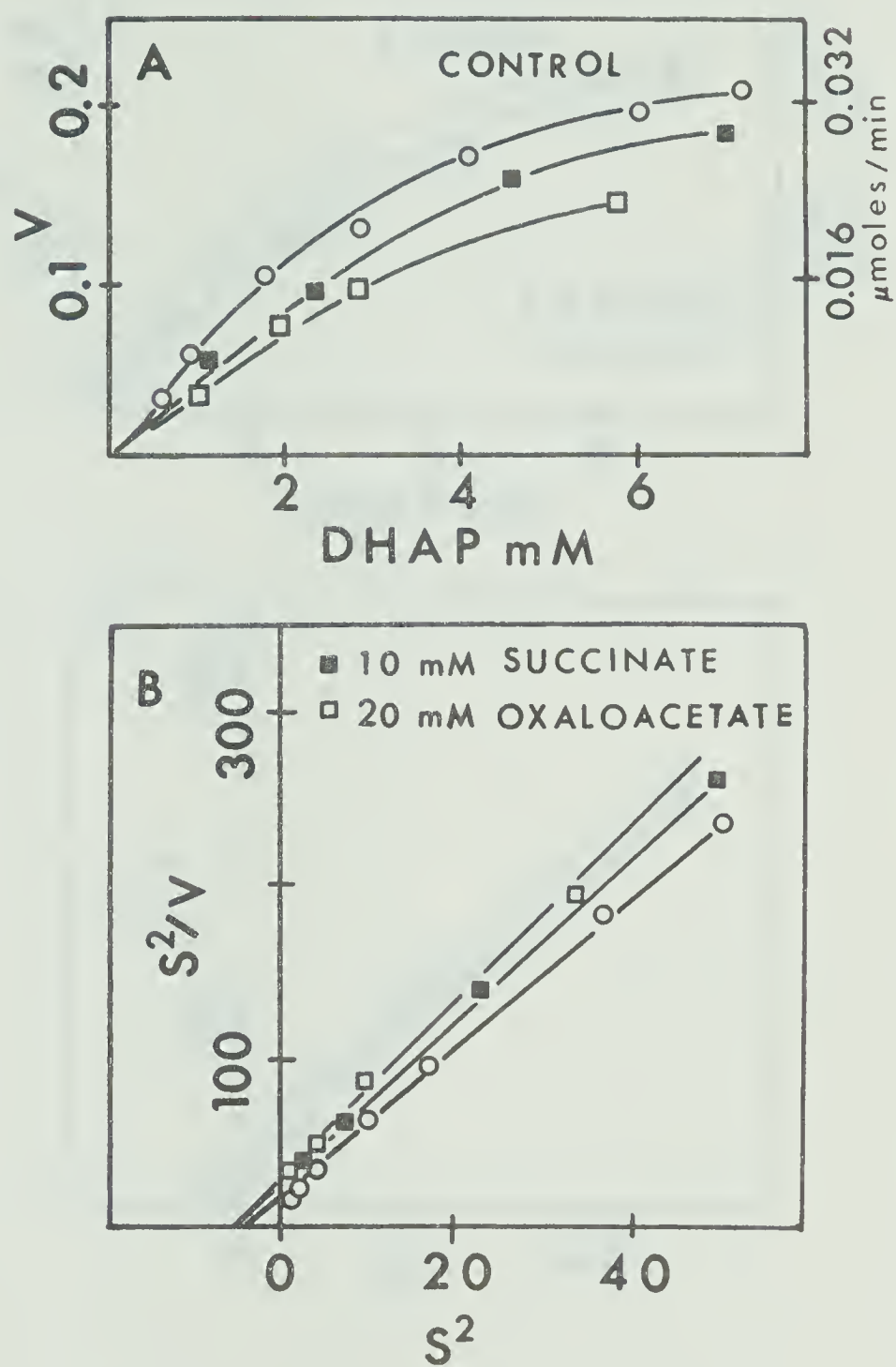


Fig. 25. Effect of succinate and oxalacetate on mosquito TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

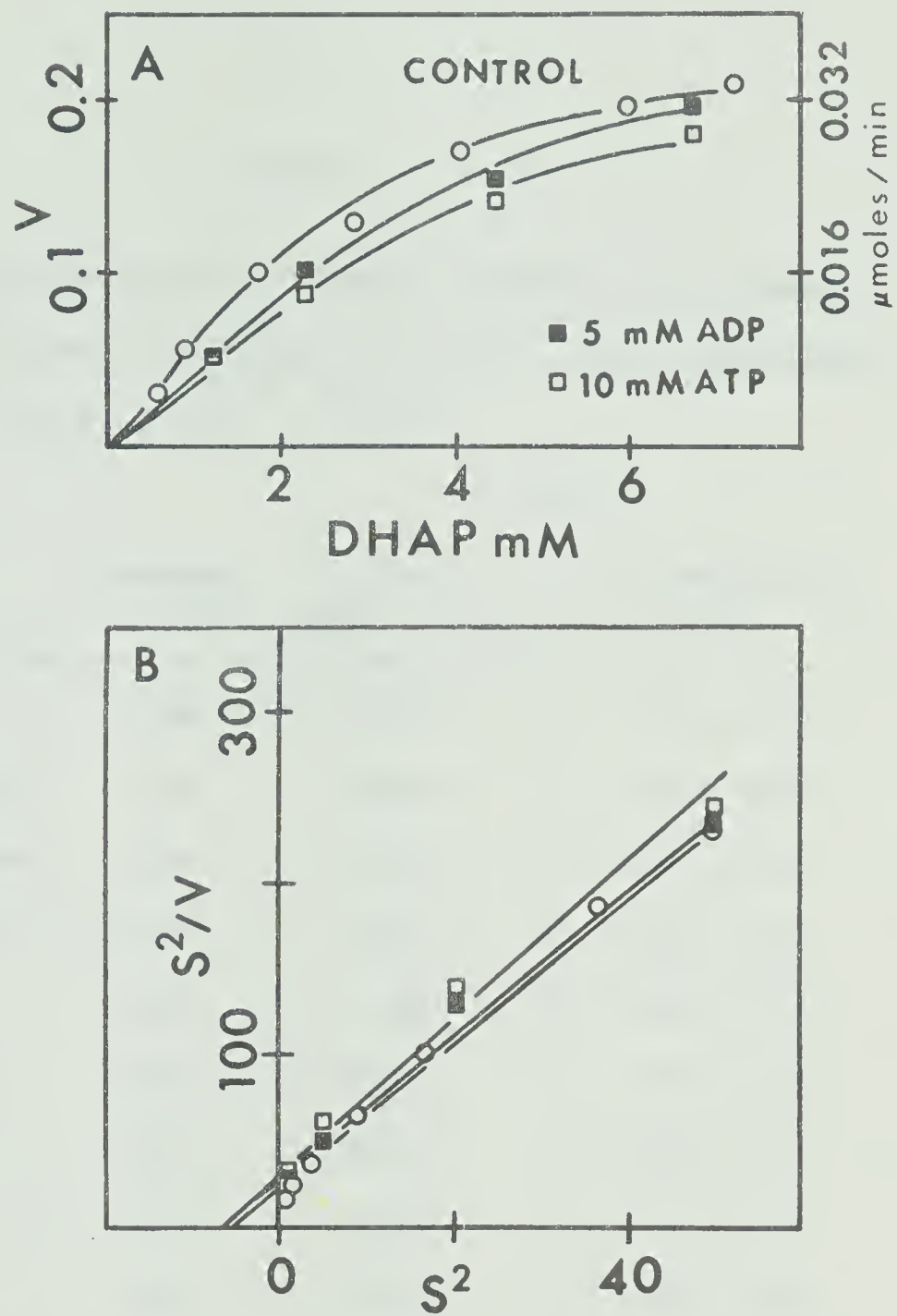


Fig. 26. Effect of ADP and ATP on mosquito TPI activity.

Modified assay method; $V = \Delta A_{340}/\text{min}$.

Table 5

Summary of the estimation of kinetic parameters for mosquito TPI; apparent K_m , and V_m by plotting S^2/V vs. S^2 ; Hill coefficients by the Hill plot. Data from Figs. 19, 22-26.

Experiment	Apparent K_m (mM)	V_m (μ moles/min)	Hill coefficient (N)
Control	1.88	0.035	1.75 ± 0.17
5 mM DL- α -Glycero-P	2.84	0.034	1.94 ± 0.06
10 mM DL- α -Glycero-P	2.91	0.028	1.81 ± 0.19
2 mM Pi	2.86	0.031	1.76 ± 0.22
4 mM Pi	3.44	0.026	1.86 ± 0.16
10 mM ATP	2.53	0.032	1.76 ± 0.32
5 mM ADP	2.32	0.034	1.83 ± 0.28
10 mM Citrate	2.68	0.034	1.74 ± 0.33
10 mM Succinate	2.25	0.032	1.86 ± 0.21
20 mM Oxaloacetate	2.45	0.032	1.78 ± 0.27
0.4 mM Folic acid	2.60	0.031	1.90 ± 0.18

Table 6

Comparison of the effect of chemicals on the conversion of DHAP to G-3-P by mosquito TPI. Each assay contained 2.88 mM DHAP, and chemical as indicated. Temp. 30 C, pH 8.0, modified assay method.

<u>Chemicals</u>		<u>% Control activity</u>
1.	DL- α -glycerophosphate (20 mM)	29.2
2.	Oxaloacetate (20 mM)	74.6
3.	Adipic acid (20 mM)	100
4.	Glutamate (20 mM)	100
5.	Glutaric acid (20 mM)	100
6.	DL-Glyceraldehyde (20 mM)	100
7.	Proline (20 mM)	100
8.	Pyruvate (20 mM)	100
9.	Pi (2 mM)	65.4
10.	ATP (2 mM)	86.5
11.	CTP (2 mM)	86.9
12.	CDP (2 mM)	86.9
13.	UTP (2 mM)	75.7
14.	GTP (2 mM)	75.7
15.	5'-AMP (20 mM)	100
16.	3', 5'-AMP (20 mM)	100

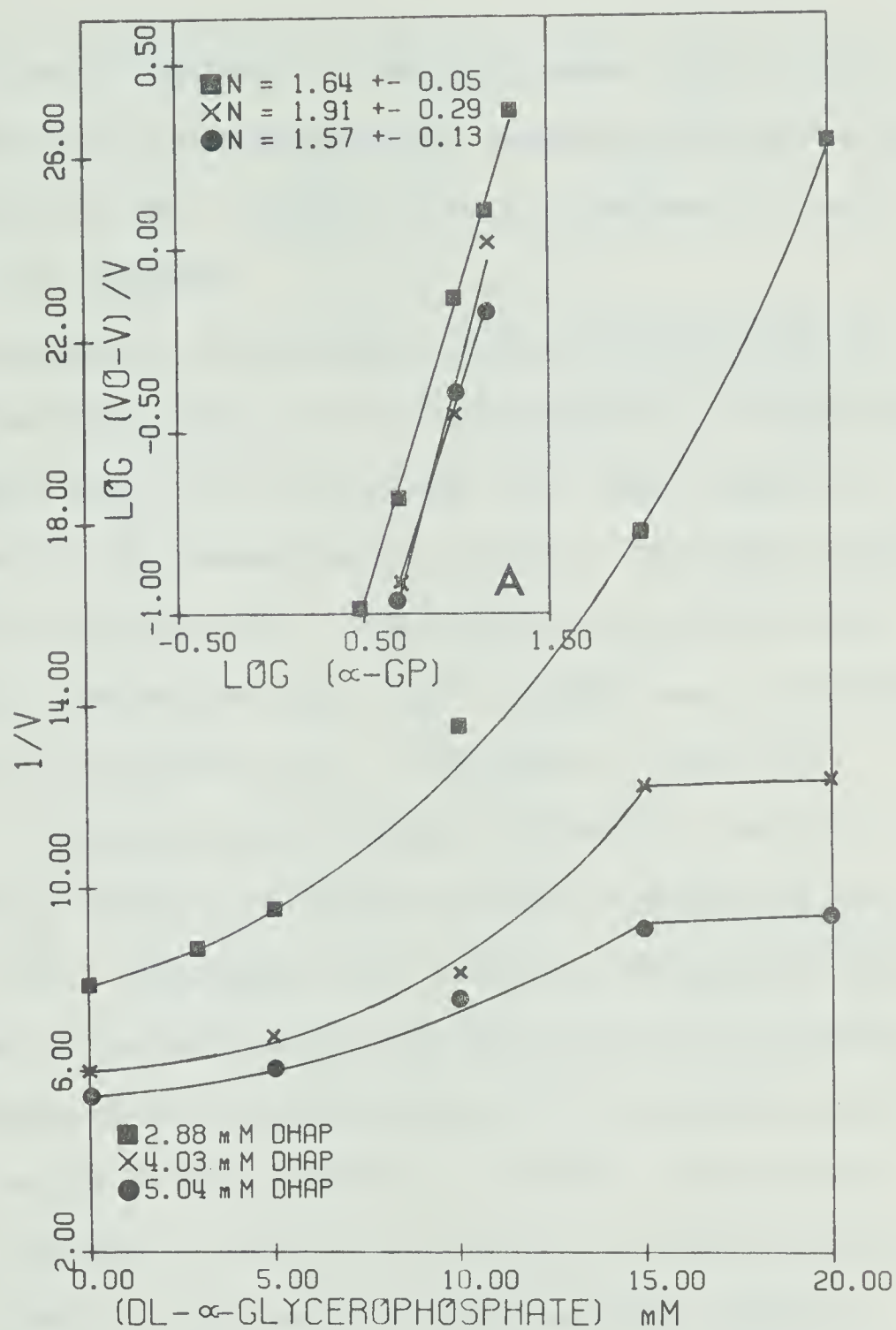


Fig. 27. Effect of varying concentrations of DL- α -glycero-phosphate on mosquito TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$; V_0 is the velocity in the absence of inhibitor.

TPI. By plotting $1/V$ against I to the 1.7th power, a K_i of 7.13 mM was obtained. β -glycerophosphate was probably a mixed inhibitor; K_i was 49.3 mM (Fig. 28). Contrary to that of α -glycero-P, non-linearity was not observed.

An intermediate in glycolysis, 3-phosphoglycerate was a competitive inhibitor, with a K_i of 31.5 mM (Fig. 29). N was 1.16 at 1.79 mM DHAP, and 1.65 at 3.14 mM DHAP (Fig. 29A). Ribose-5-phosphate, part of the pentose shunt, was also a competitive inhibitor with a K_i of 24.1 mM (Fig. 30). N decreased from 1.94 at 2.1 mM DHAP to 1.08 at 2.88 mM DHAP (Fig. 30A). Arsenate was an inhibitor, giving a K_i of 29.37 mM (Fig. 31). N was about 1.2 (Fig. 31A).

IAP (1-hydroxy-3-iodo-2-propanone phosphate), from 50 to 200 μ M, inhibited mosquito TPI in the presence of DHAP, 0.96 to 4.80 mM (Fig. 32). The extent of IAP inhibition increased as DHAP concentrations in the mixture were increased from 0.96 to 2.60 mM; further increases in DHAP concentration to 3.60 or 4.80 mM resulted in a marked decline in IAP inhibition of the TPI. An uncritical treatment of the data by the Hill plot yields the N and I_{50} values presented in table 7. The results of this experiment suggest a heterotropic co-operativity i.e. the promotion of binding of IAP by DHAP concentration was increased from 0.96 to 2.60 mM. However at higher DHAP concentrations one of the sites available for IAP was protected by DHAP.

Interpretation of the IAP inhibition data is difficult

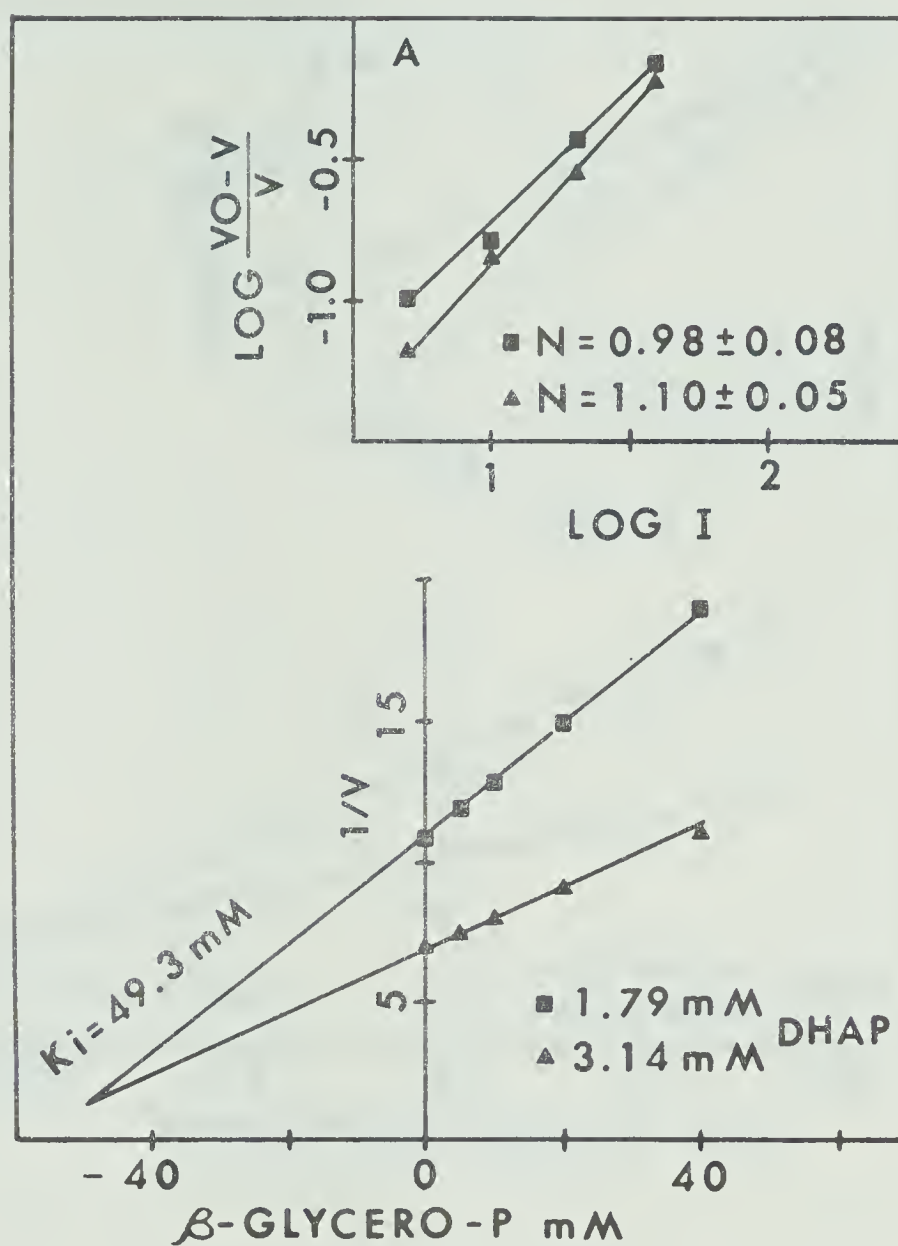


Fig. 28. Effect of β -glycerophosphate on mosquito TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

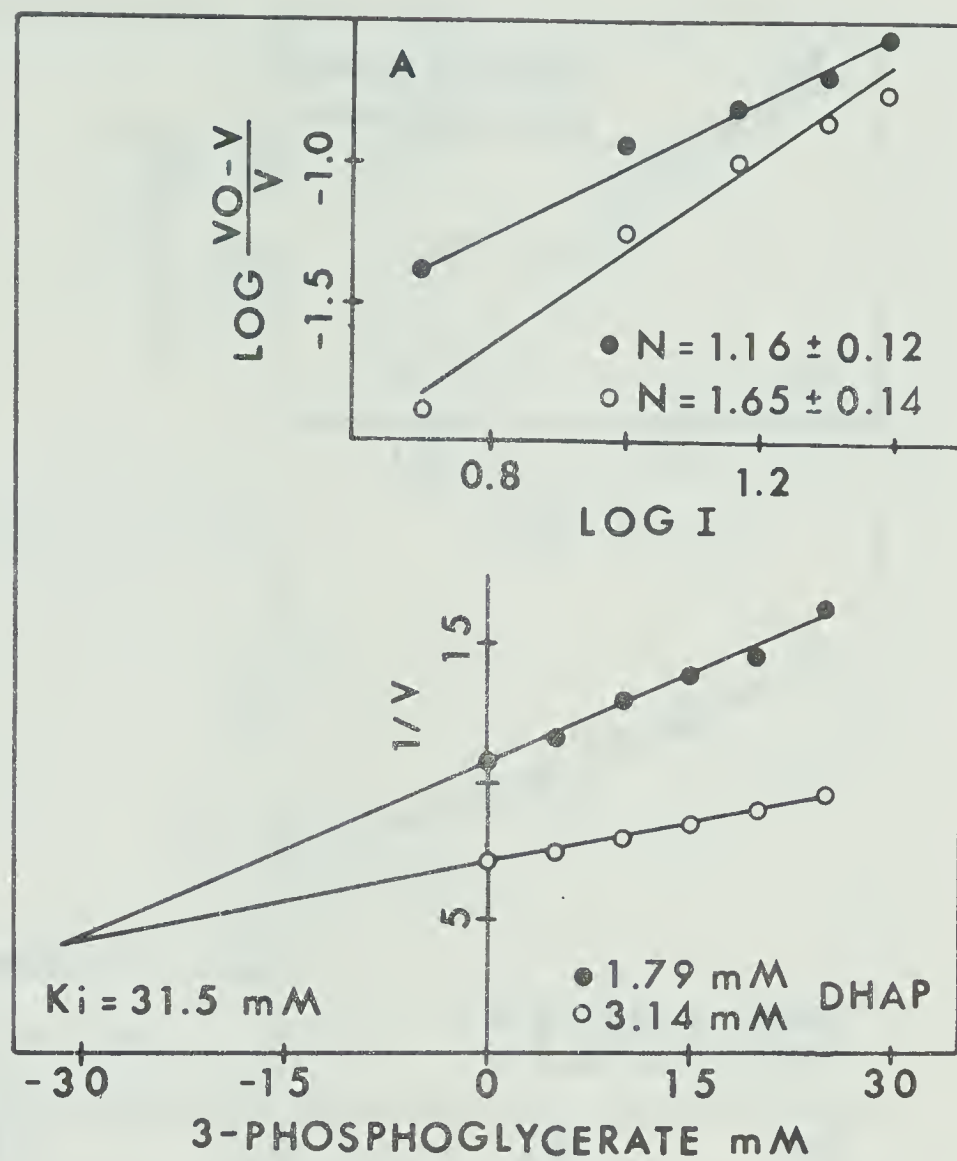


Fig. 29. Effect of 3-phosphoglycerate on mosquito TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min.}$

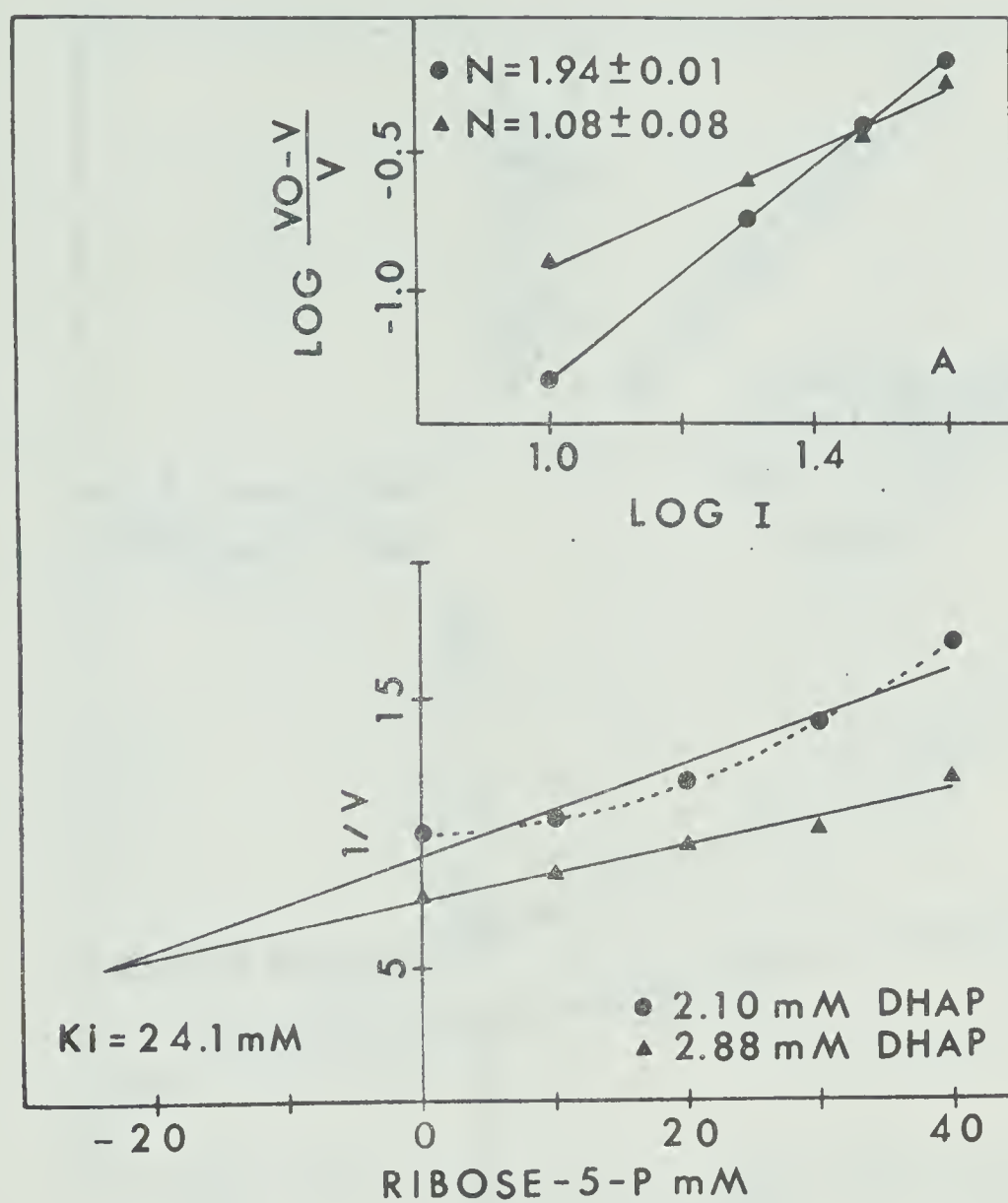


Fig. 30. Effect of ribose-5-phosphate on mosquito TPI activity.

Modified assay method; $V = \Delta A_{340}/\text{min}$.

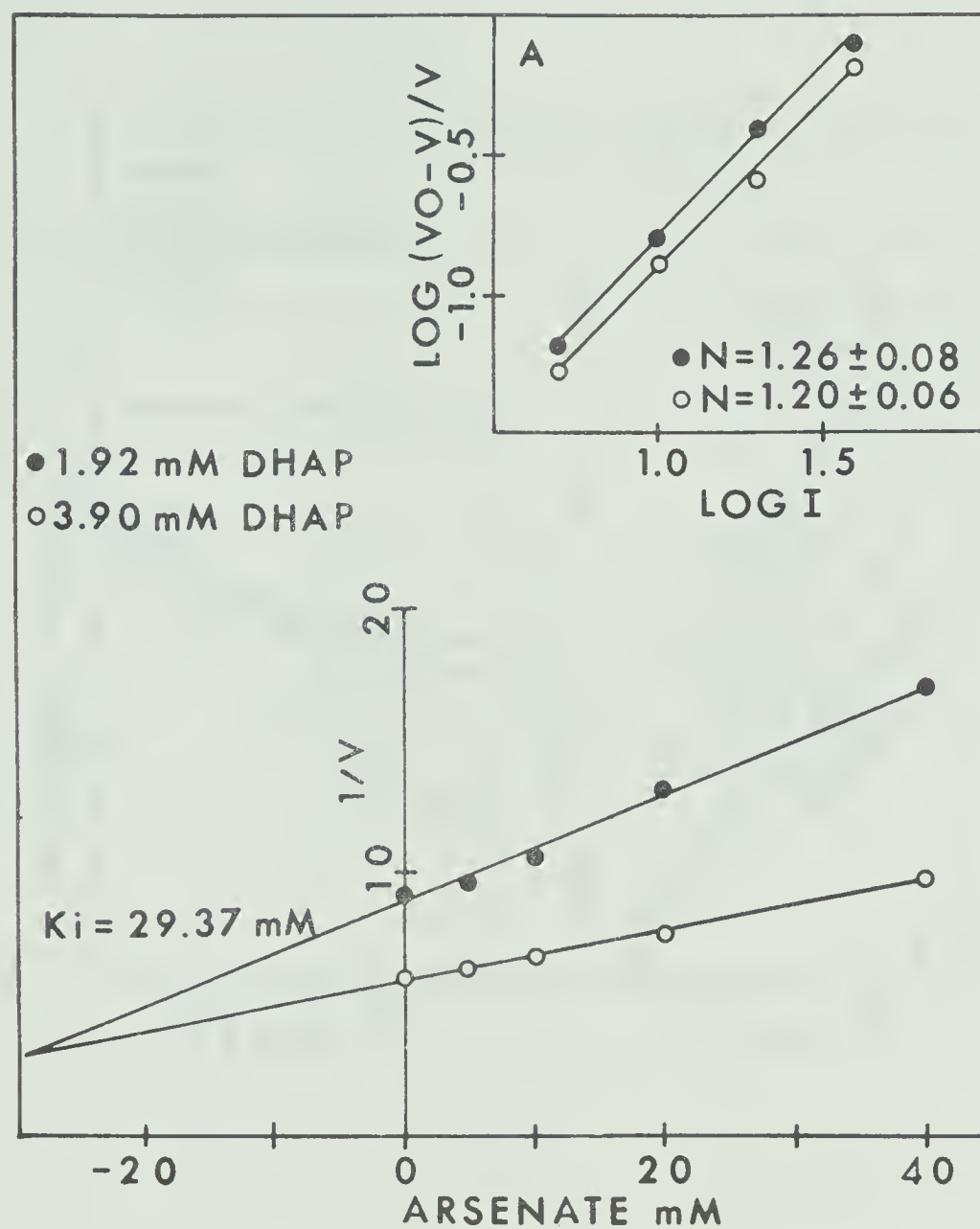


Fig. 31. Effect of arsenate on mosquito TPI activity.

Modified assay method; $V = \Delta A_{340}/\text{min}$.

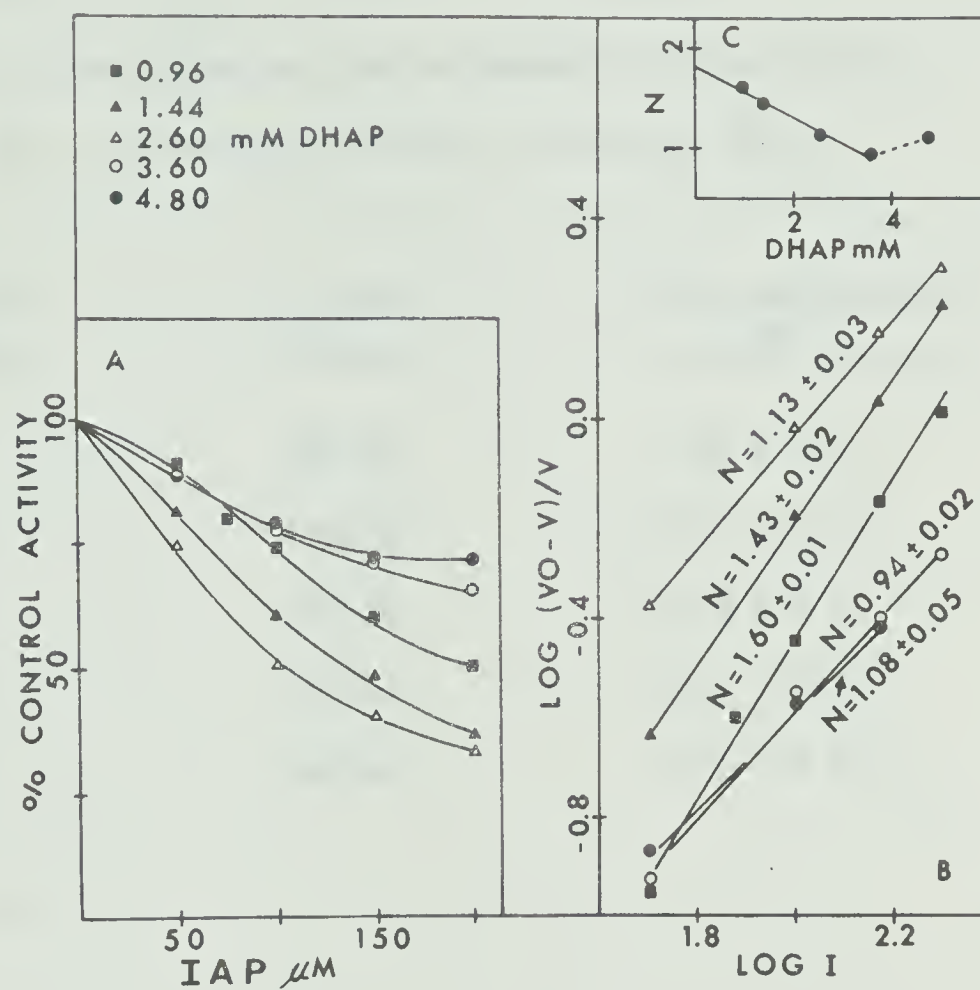


Fig. 32. Effect of 1-hydroxy-3-iodo-2-propanone phosphate (IAP) on mosquito TPI activity. Inhibitory pattern at 3.6 mM DHAP is not statistically different from that at 4.8 mM DHAP by T-test ($P > 0.05$). Modified assay method; $V = \Delta A_{340}/\text{min}$.

Table 7

Summary of the effect of IAP on mosquito TPI activity.

Data from Fig. 32. I_{50} estimated by Hill plot (Fig. 32B).

<u>DHAP conc. (mM)</u>	<u>I_{50} (μM)</u>	<u>Hill coefficient (N)</u>
0.96	189.99	1.60 ± 0.01
1.44	139.84	1.43 ± 0.02
2.60	106.32	1.13 ± 0.03
3.60	413.94	0.94 ± 0.02
4.80	348.44	1.08 ± 0.05

and not analyzable by the Dixon plot because IAP is an irreversible inhibitor of TPI (Hartman, 1968) and thus the concentration of active TPI in the reaction mixture was continuously declining during the assay.

The effect of pH on the inhibition of mosquito TPI by Pi

The inhibitory effect of Pi, glycerol-P, citrate, oxaloacetate, folic acid, succinate, arsenate, ribose-5-phosphate, 3-phosphoglycerate and β -glycerophosphate suggests that the binding of ligands to TPI involves the 2 oxyanions on these ligands. The inability of the tested mono-carboxylic acids to inhibit mosquito TPI augments this hypothesis. The interchange distances, in Angstroms (A), between the 2 oxyanions of the following chemicals are: 2.42 for oxalate; 4.75 for the trans-isomer and 3.01 for the cis-isomer of succinate; 5.83 for trans-isomer and 4.84 for the cis-isomer of glutarate; 6.87 for the trans-isomer of adipic acid, which is a 6-carbon dicarboxylic acid (Webb, 1966). The interchange distance between the 2 oxyanions of the HPO_4^{2-} is 3.08 A (Bowen, 1958). Since oxalate is an inhibitor for rabbit TPI (Wolfenden, 1969), it seems that the binding of inhibitors onto the enzyme occurs within a certain limit of the interchange distances between the 2 oxyanions, probably between 2.42 and 3.08 A.

Wolfenden (1969) found a whole series of inhibitors for rabbit TPI with G-3-P as substrate. All these inhibitors were either phosphates, or dicarboxylic acids. He attributed the effect of these inhibitors to their ability to form transition state analogs for TPI. Rose and O'Connell (1969) proposed that the inactivation

of rabbit TPI by glycidol phosphate (1,2-epoxipropanol-3-P) was due to the ester linkage formation.

Since the concentration of the dibasic species of Pi, HPO_4^{2-} , depends on the pH of the medium, lowering the pH of the assay solution will lessen the concentration of HPO_4^{2-} . An increase in the apparent K_i should be observed when the pH is lowered. This would then support the hypothesis that the 2 oxyanions are involved in the binding with TPI.

At pH 8.0, the apparent K_i was 0.8 mM Pi (Fig. 33A). Non-linearity was observed at 0.88, 1.76 and 2.37 mM DHAP. There was saturation of TPI by Pi, but this was abolished at 3.52 mM DHAP. With the Hill plot, N decreased from 1.75 to 1.01 with increasing DHAP concentrations (Fig. 33B). Replot of N against DHAP concentration gave a value of 2 as the maximum by regression (Fig. 33C). Atkinson (1966) attributed the non-integral values of N to the existence of a mixture of molecules at varying degrees of saturation. In the presence of an inhibitor, the increase of N with each decreasing substrate concentration was interpreted as a result of homotropic cooperativity by the inhibitor itself (Madsen and Shechosky, 1967). At pH 7.0, the apparent K_i was 0.85 mM Pi (Fig. 34). At pH 6.5, the apparent K_i was 2.67 mM Pi (Fig. 35), and at pH 6.0, 4.18 mM Pi (Fig. 36). The pattern of inhibition shifted from a mixed type to non-competitive inhibition through the gradual lowering of the common intercepts of the regression lines. The total effect of changing the pH on the inhibition of mosquito TPI activity is summarized in Table 8. When

the Pi concentrations were converted to the HPO_4^{2-} concentrations, and plotted onto the Dixon plot, the intrinsic K_i varied. But the variation was rather slight from pH 7.0 to 6.0. The intrinsic K_i at pH 7.0 was approximately the same as that at pH 6.0, 0.33 and 0.30 mM respectively. The apparent K_i became asymptotic at pH 7.0, at which the Pi is about 40% HPO_4^{2-} (Fig. 37A, B). This indicates that maximal control by Pi is readily achieved at physiological pH. Measured before and after flight, the pH of insect haemolymph was within the same range, 6.9-7.1 (Friedman, 1959).

Concomitant changes, such as conformational changes in the enzyme, an increase or decrease in the apparent K_m values, or the availability of certain ionizable groups on the enzyme moiety, may affect the $K_i(s)$ in question. The influence of pH on dimer-monomer equilibrium cannot be overlooked (Gazith *et al.*, 1968). Because the apparent K_i did increase from pH 7.0 to 6.5, and the intrinsic K_i remained approximately equal, the involvement of the 2 oxyanions of Pi at the binding site thus seems necessary.

The effect of temperature on the inhibition of mosquito

TPI by Pi

Since temperature often affects the equilibrium of an ionized species of a chemical (Dawes, 1964), and since the body temperature of insects changes during flight, the effect of temperature on the inhibition of mosquito TPI by Pi was studied at pH 7.0. The inhibition by Pi at 30 C was already presented Fig. 34. Non-competitive inhibition occurred at 25 C, and the K_i was 4.37 mM (Fig. 38). N was about

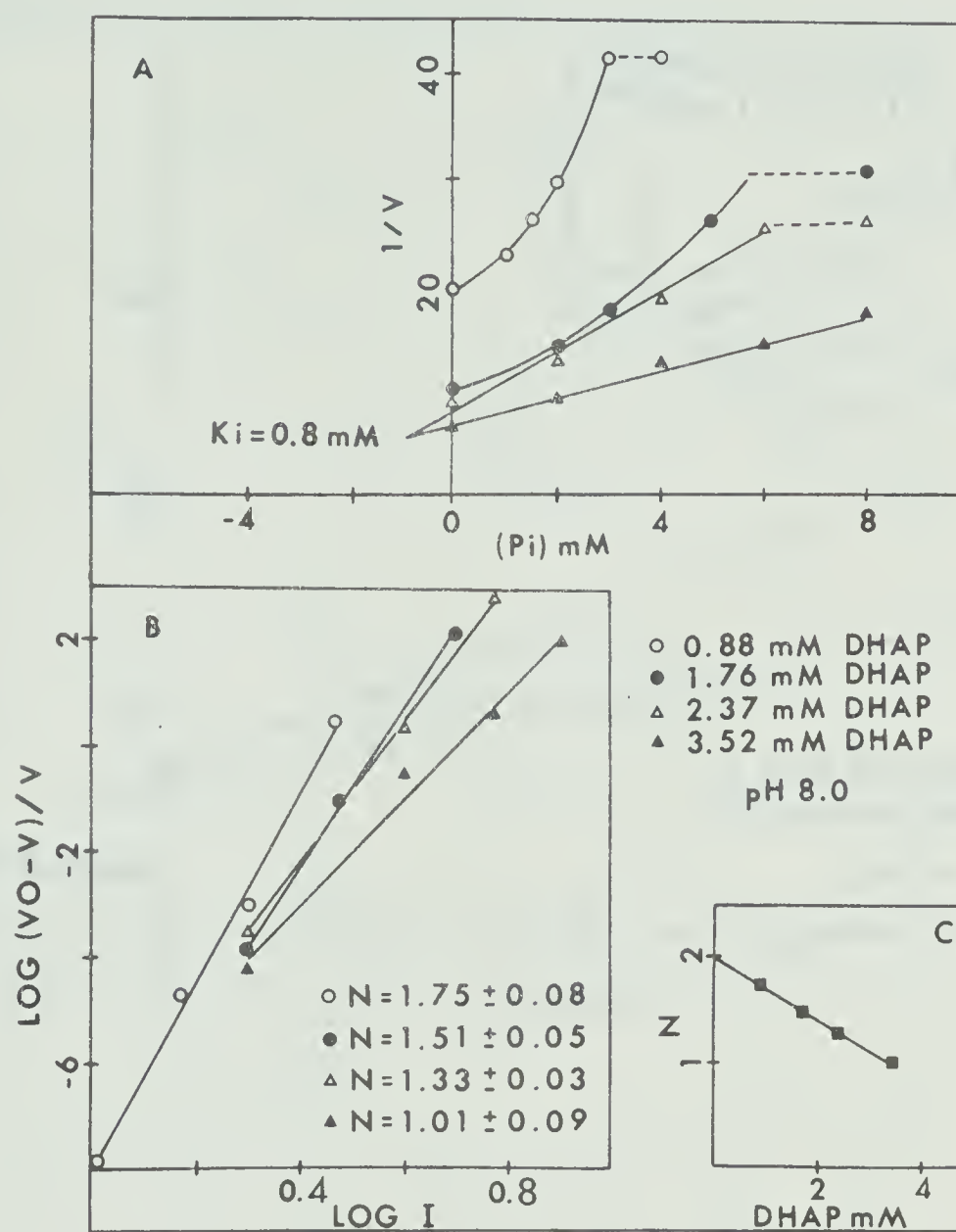


Fig. 33. Effect of Pi on mosquito TPI activity at pH 8.0. Assay procedure as described in methods. Modified assay method; $V = \Delta A_{340}/\text{min}$.

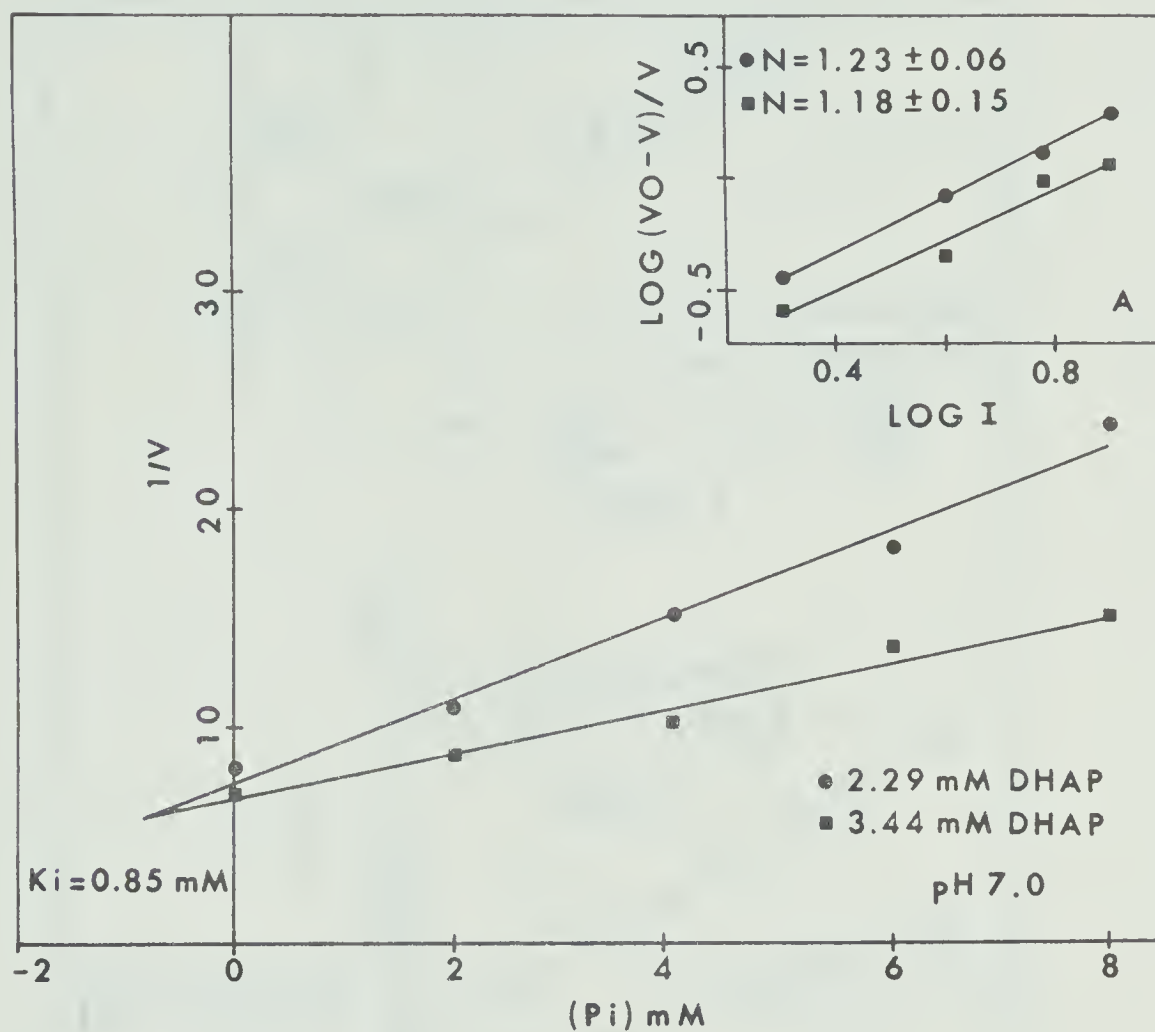


Fig. 34. Effect of Pi on mosquito TPI activity at pH 7.0. Modified assay method; reaction stopped by 0.4 ml of 5% TCA after 3 min of incubation. From 0 mM to 6 mM Pi, 2 drops of 2.5 N NaOH required to neutralize the assay solution; at 8 mM Pi, one drop of 2.5 N NaOH required. $V = \Delta A_{340}/\text{min}$.

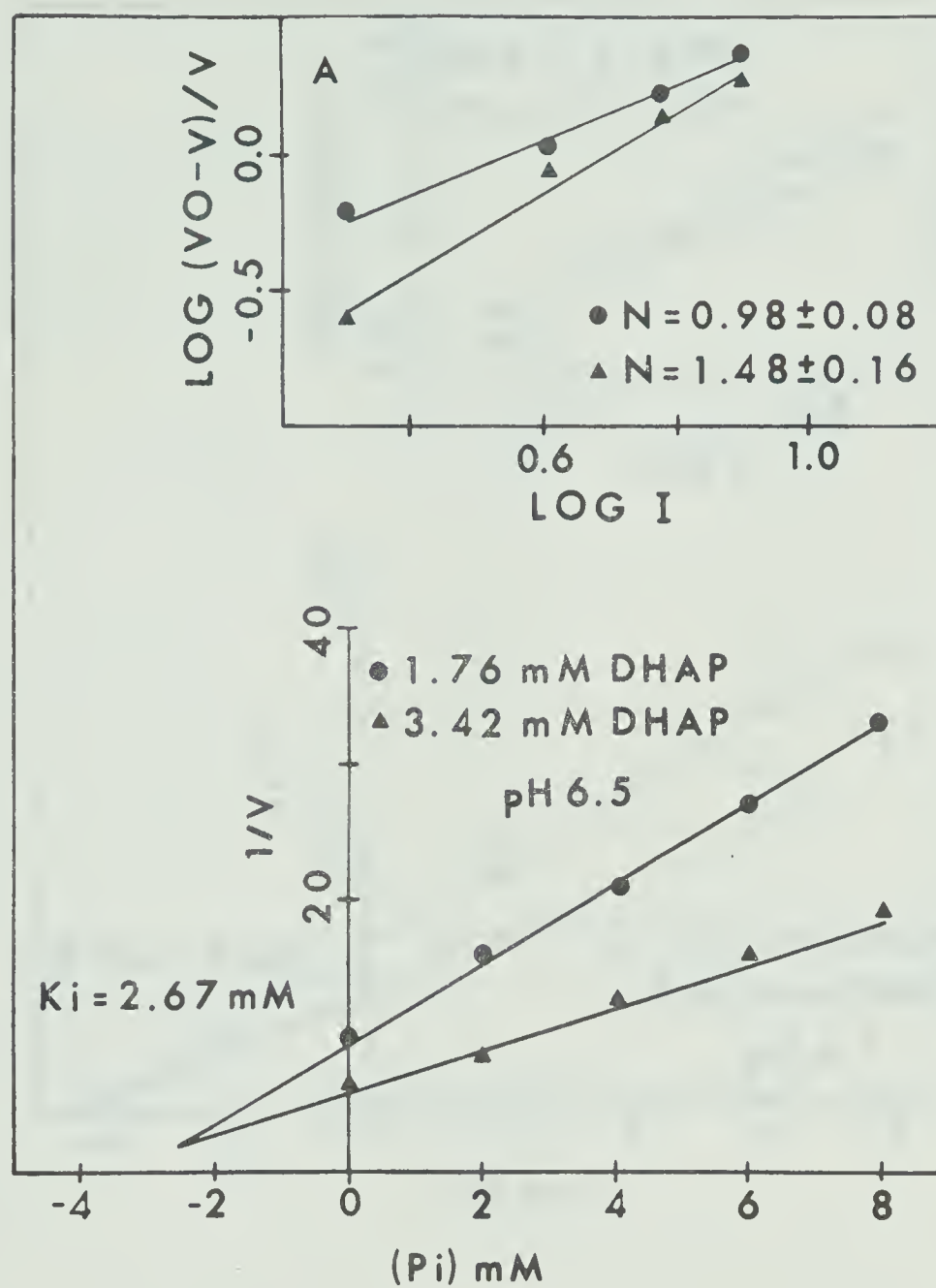


Fig. 35. Effect of Pi on mosquito TPI activity at pH 6.5.

Method as described in Fig. 34; $V = \Delta A_{340}/\text{min}$.

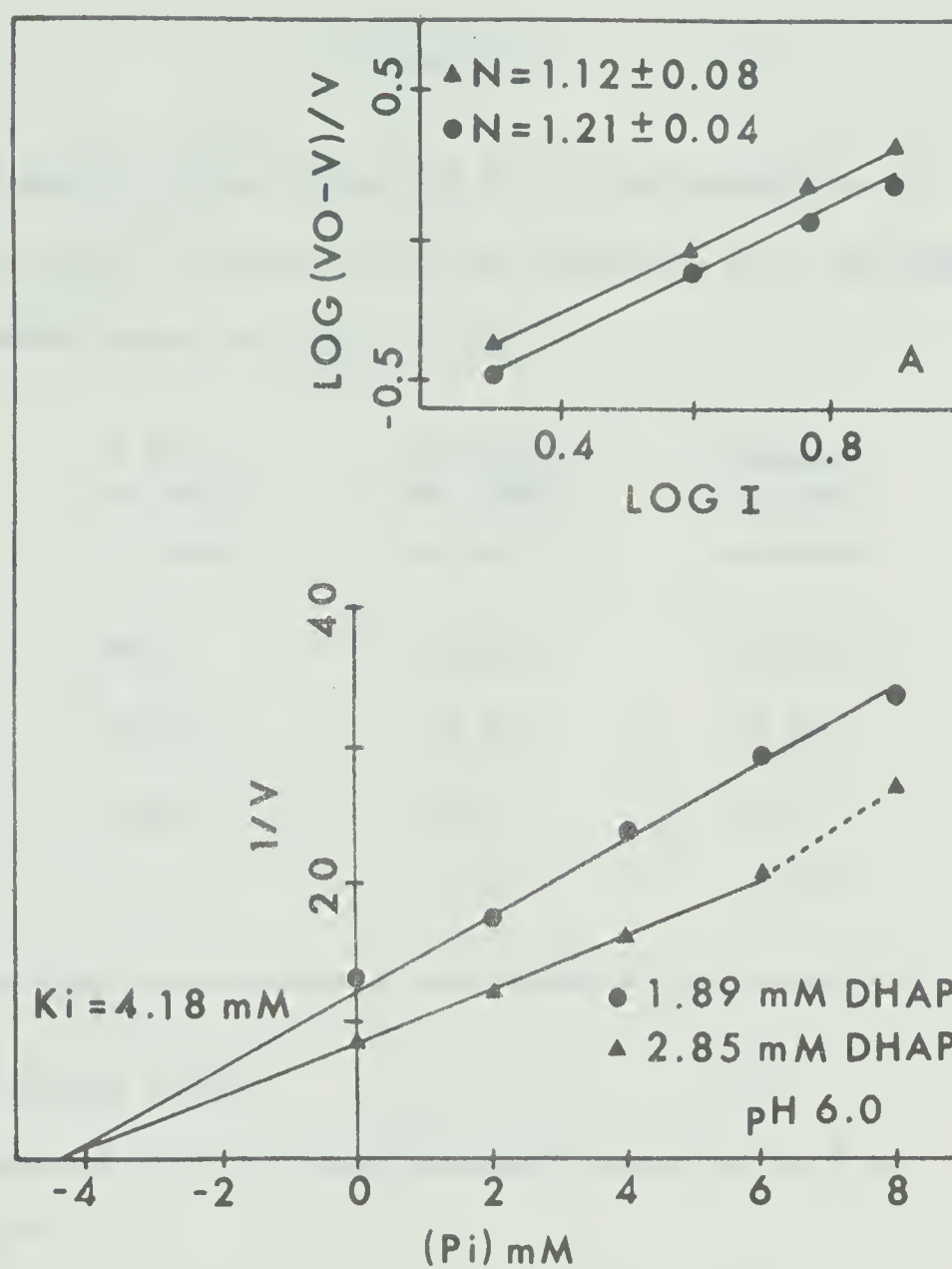


Fig. 36. Effect of Pi on mosquito TPI activity at pH 6.0.

Method as described in Fig. 34; $V = \Delta A_{340}/\text{min.}$

Table 8

Summary of the effect of Pi on the conversion of DHAP to G-3-P by mosquito TPI at different pH. The same data also appear in Figs. 33-36.

pH	% Pi* in HPO_4^{2-}	Intrinsic Ki (mM) ⁺	Apparent Ki (mM)
8.0	86.3	0.92	0.80
7.0	38.68	0.33	0.85
6.5	16.63	0.44	2.27
6.0	5.93	0.30	4.20

*From Albert (1965)

⁺Calculated with Pi concentrations corrected to % in HPO_4^{2-}

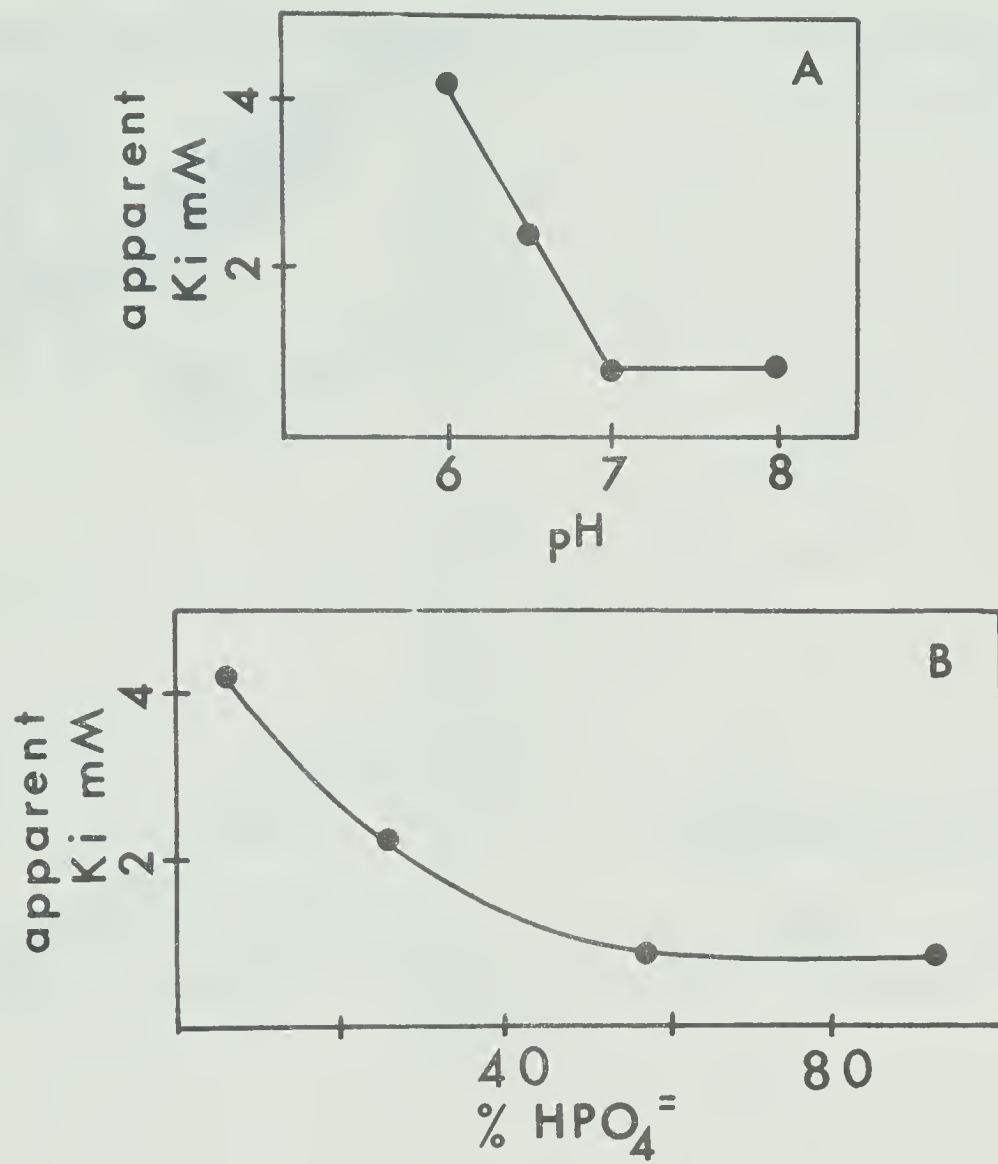


Fig. 37. Replots of K_i obtained from Figs. 33-36.

A. Apparent K_i vs. pH. B. Apparent K_i vs. % HPO_4^{2-} .

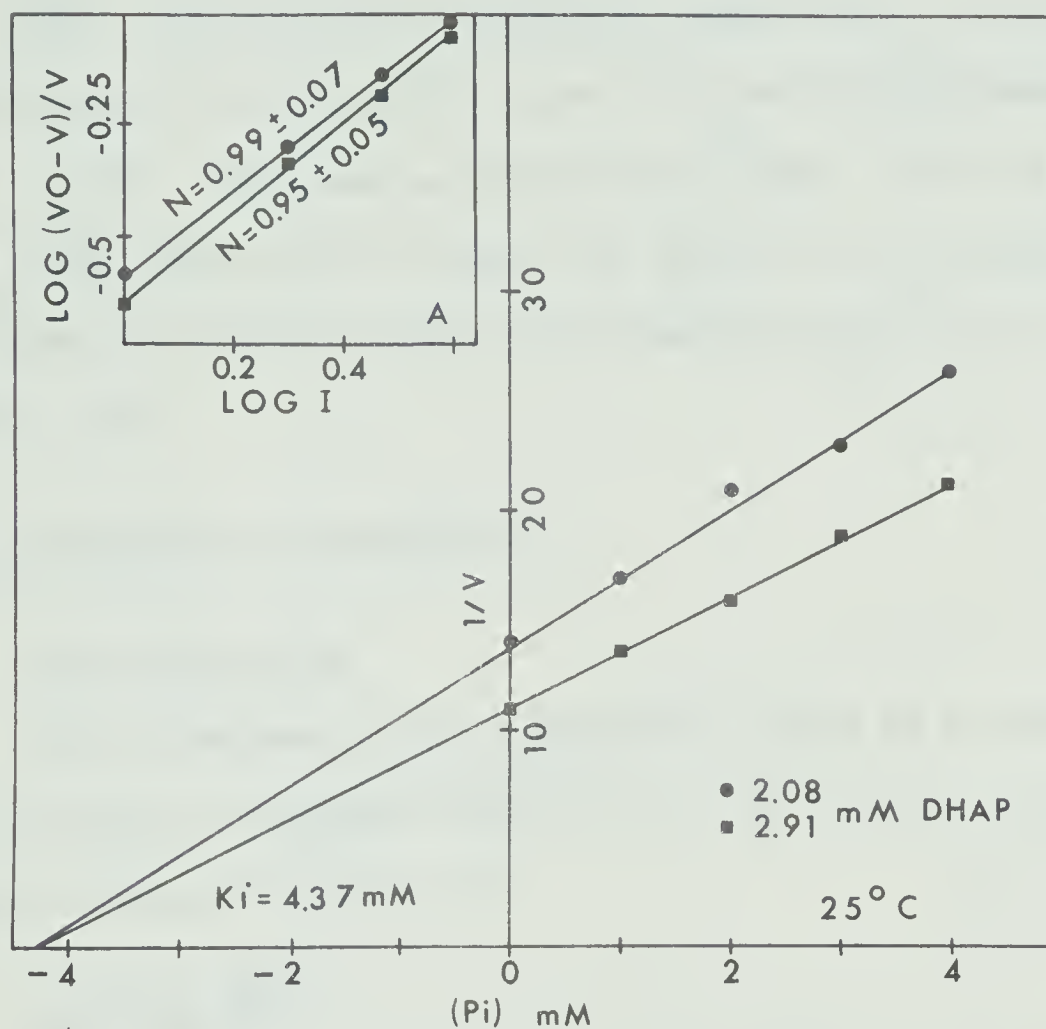


Fig. 38. Effect of Pi on mosquito TPI activity at 25°C , pH 7.0. Method as described in Fig. 34; $V = \Delta A_{340}/\text{min}$.

1 (Fig. 38A). The pattern of inhibition became mixed, or possibly competitive, at 30 C and 40 C; K_i was 0.85 and 0.27 mM respectively (Figs. 34, 39). There was an indication of higher orders of binding with N values from 1 to 1.57 (Fig. 34A, 39A). The activation energy for binding was +32.69 kcal/mole by Arrhenius plot of $\text{Log } 1/K_i$ against $1/T$ (Fig. 40).

Kinetic Properties of Housefly TPI

The effect of pH

The pH optimum for the housefly TPI was at pH 8.8 when G-3-P was used as substrate (Fig. 41). The pH activity profile was similar to that of mosquito TPI.

G-3-P to DHAP

With G-3-P as substrate, substrate inhibition was observed (Fig. 42A). But there was no apparent deviation from the simple Michaelis-Menton equation for the data points prior to substrate inhibition, and Hill plot of the same data gave an N of 1 (Fig. 42B). The K_m was 1.16 mM G-3-P; 95% confidence limits from 0.9 to 1.44 mM. Substrate inhibition was observed for the rabbit TPI also (Fig. 45). An allosteric site was suggested for substrate inhibition of rabbit TPI (Snyder and Lee, 1966).

DHAP to G-3-P

The kinetic behavior of housefly TPI was similar to that of mosquito TPI with DHAP as substrate. Assayed by the coupling

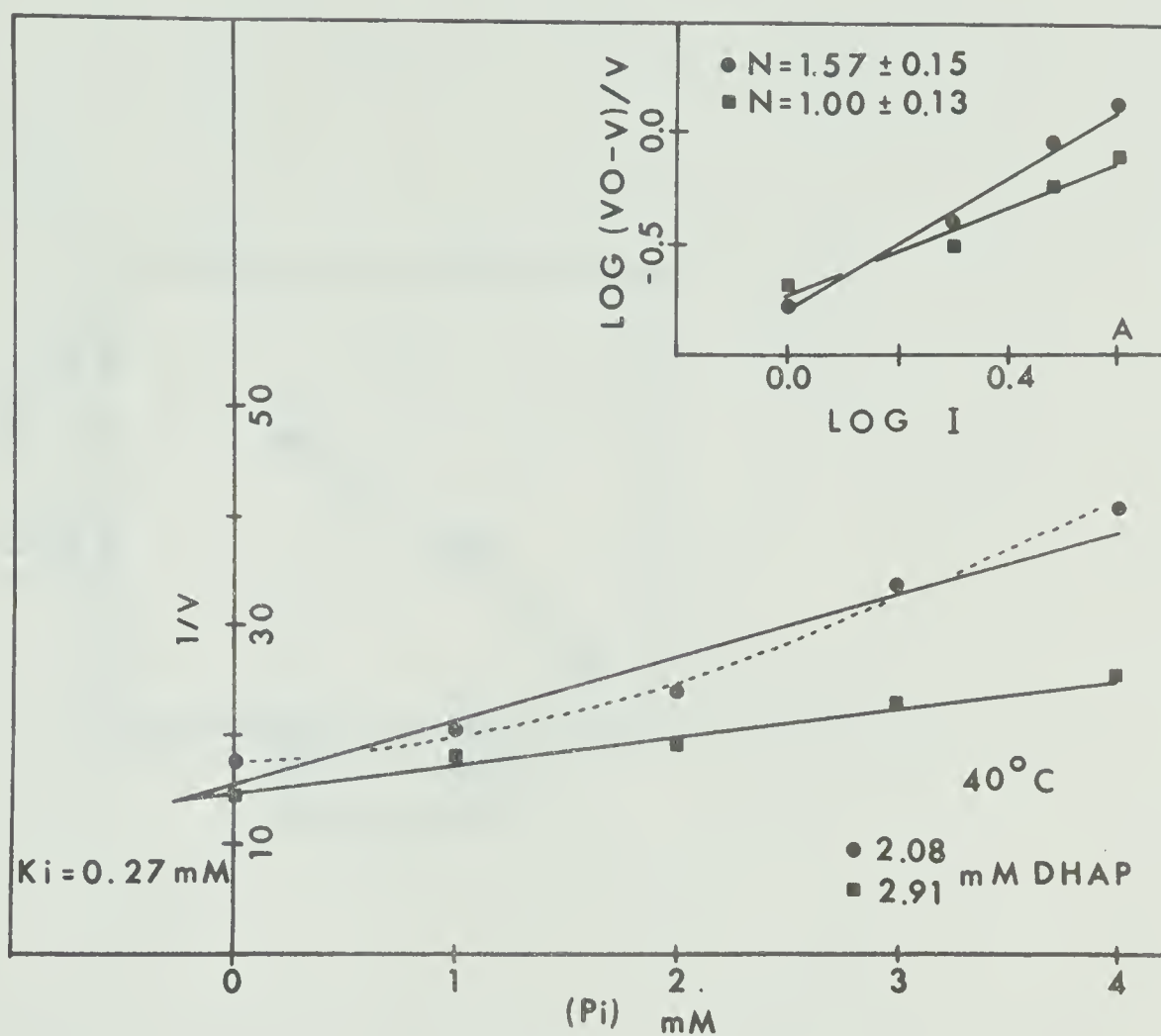


Fig. 39. Effect of P_i on mosquito TPI activity at 40°C , pH 7.0. Method as described in Fig. 34, 28.3 ng TPI; $V = \Delta A_{340}/\text{min}$.

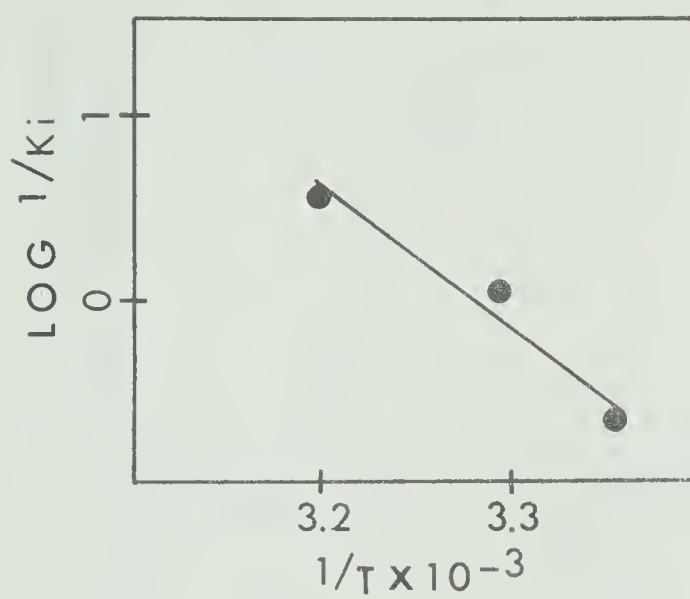


Fig. 40. Arrhenius plot of $\text{Log } 1/K_i$ vs. $1/T$. Data from Figs. 34, 38 and 39.

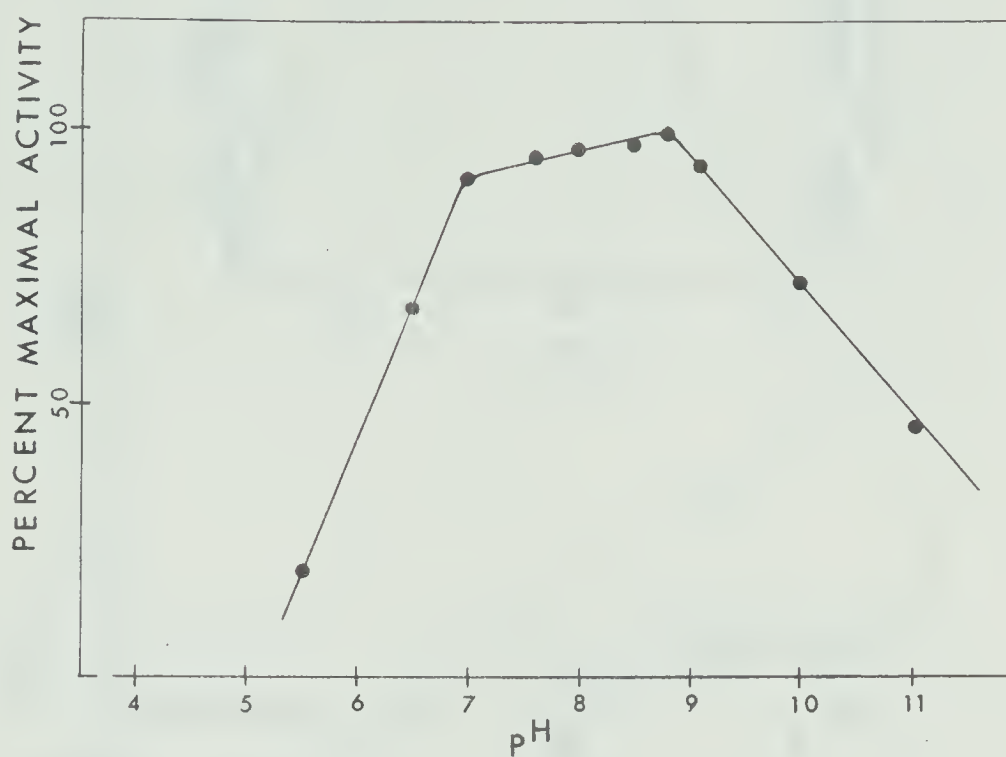


Fig. 41. Effect of pH on the conversion of G-3-P (1.2 mM) to DHAP by housefly TPI. 50 mM TEA, 5 mM EDTA, temp. 30 C.

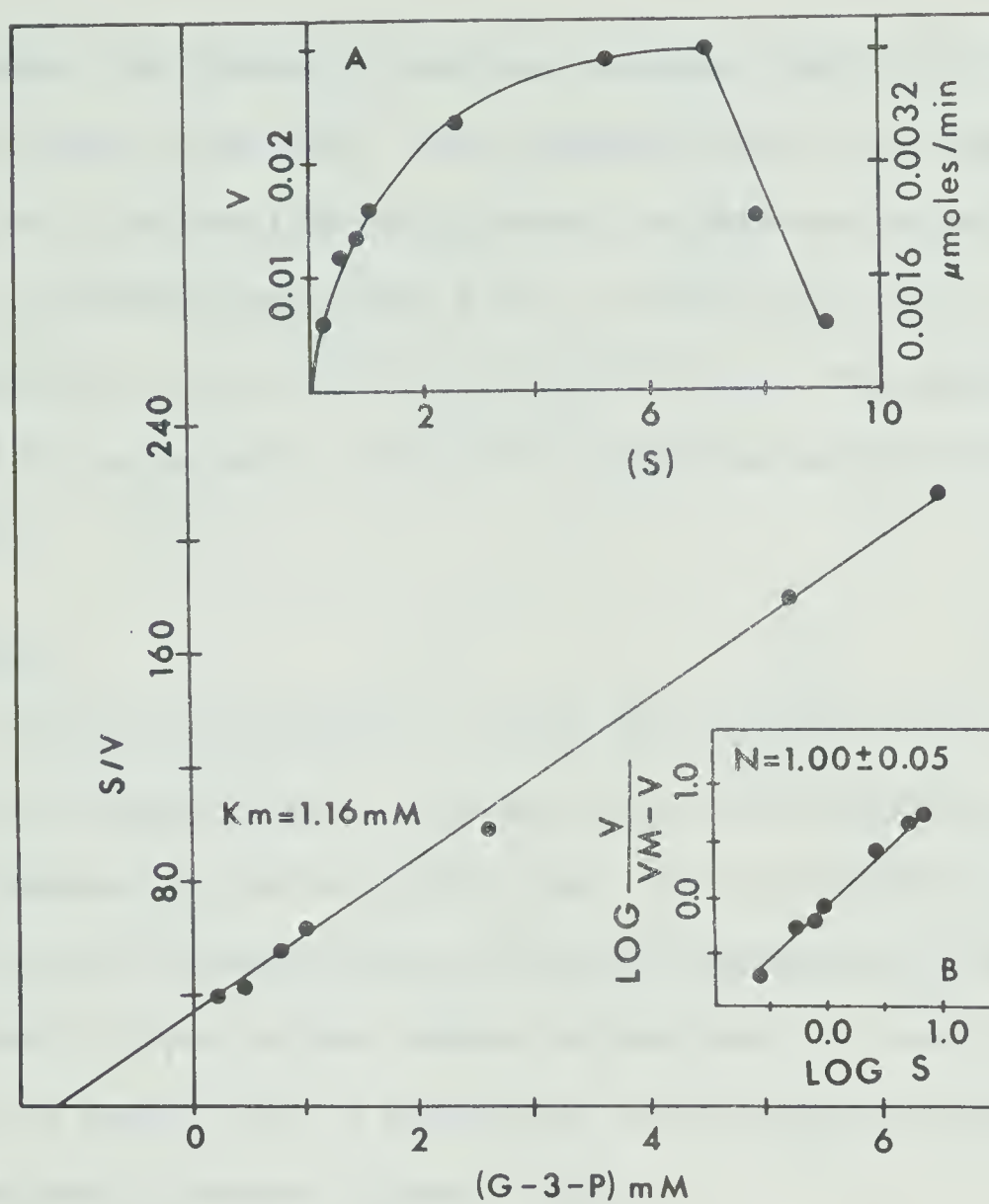


Fig. 42. Effect of G-3-P concentrations on housefly TPI activity. 0.2 ng TPI, 50 mM TEA, 5 mM EDTA, pH 8.8, 30 C, $V = \Delta A_{340}/\text{min}$.

enzyme method, the Michaelis curve was sigmoidal (Fig. 43). The apparent K_m was 1.27 mM DHAP, with confidence limits from 0.89 to 1.57 mM. With the modified assay method, the apparent K_m was 1.1 mM DHAP; confidence limits from 0.94 to 1.41 mM (Fig. 44). Using the observed V_m , an N of 2.08 ± 0.21 was obtained. This agreed well with the estimated N , 1.86 ± 0.12 , using the calculated V_m (Fig. 44B).

Discussion I

The kinetic properties of insect TPI suggests that it is a regulatory enzyme having an allosteric site. The activity of insect TPI is attenuated by various metabolites. The allosteric site is revealed by the experiments with IAP and the experiments with P_i . The allosteric nature of this enzyme bestows upon the insects a control at a branch point of metabolism, aptly evolved for their special metabolic feature--glycero-P shunt.

After blood meals, the rate of glycogen synthesis in mosquitoes was less than half the rate of fat synthesis (Van Handel, 1965). Van Handel (1965) suggested that protein and sugar lead to triglycerides through a common rate-limiting precursor. It is conceivable that this rate-limiting precursor occurs via the glycero-P shunt by regulating the TPI activity. Mosquitoes and some other flies use carbohydrates for flight (Clements, 1955; Hocking, 1953). But the mosquito can convert the fat reserves to carbohydrates for flight after the exhaustion of the original carbohydrates (Clements, 1955).

The mitochondria of *Aedes aegypti* oxidized all the Krebs

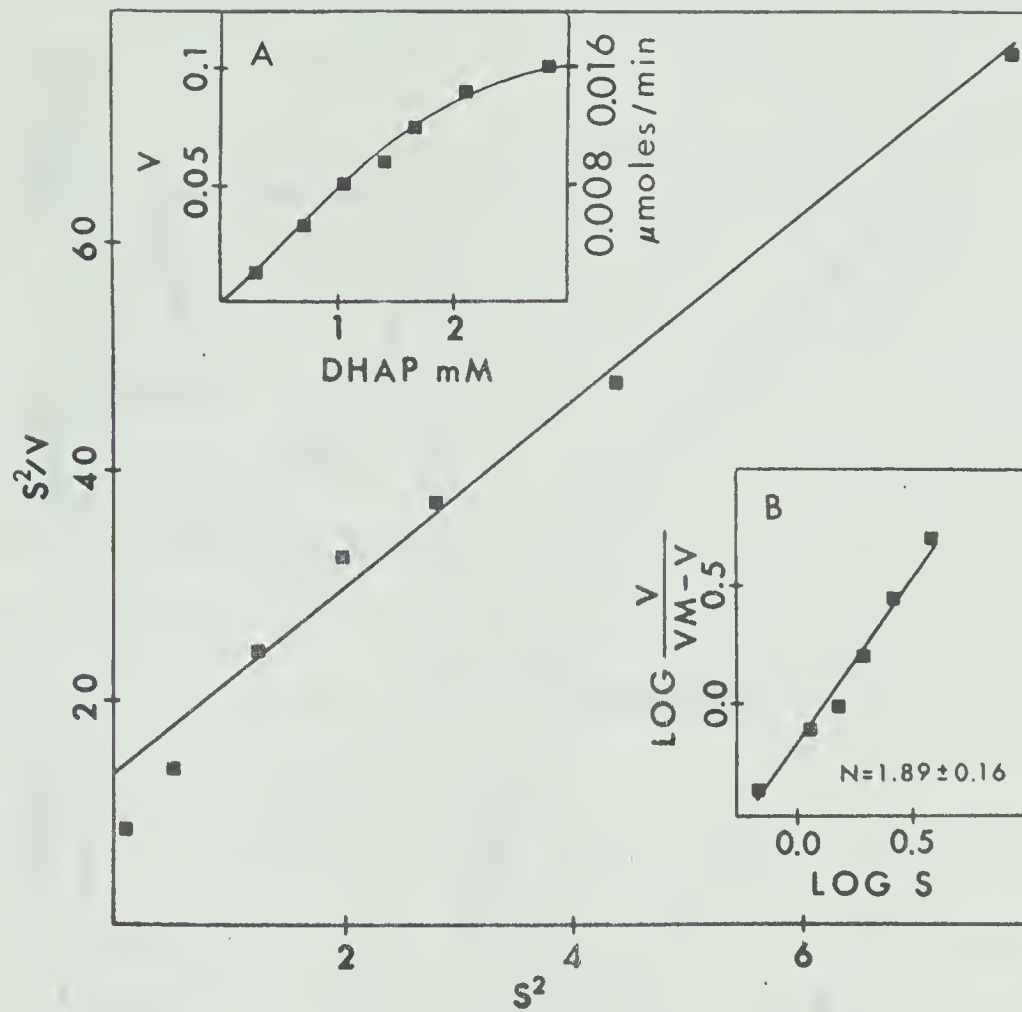


Fig. 43. Effect of DHAP concentrations on housefly TPI activity by the coupling enzyme method. Temp. 30 C, pH 8.8, 6 ng TPI, $V = \Delta A_{340}/\text{min}$.

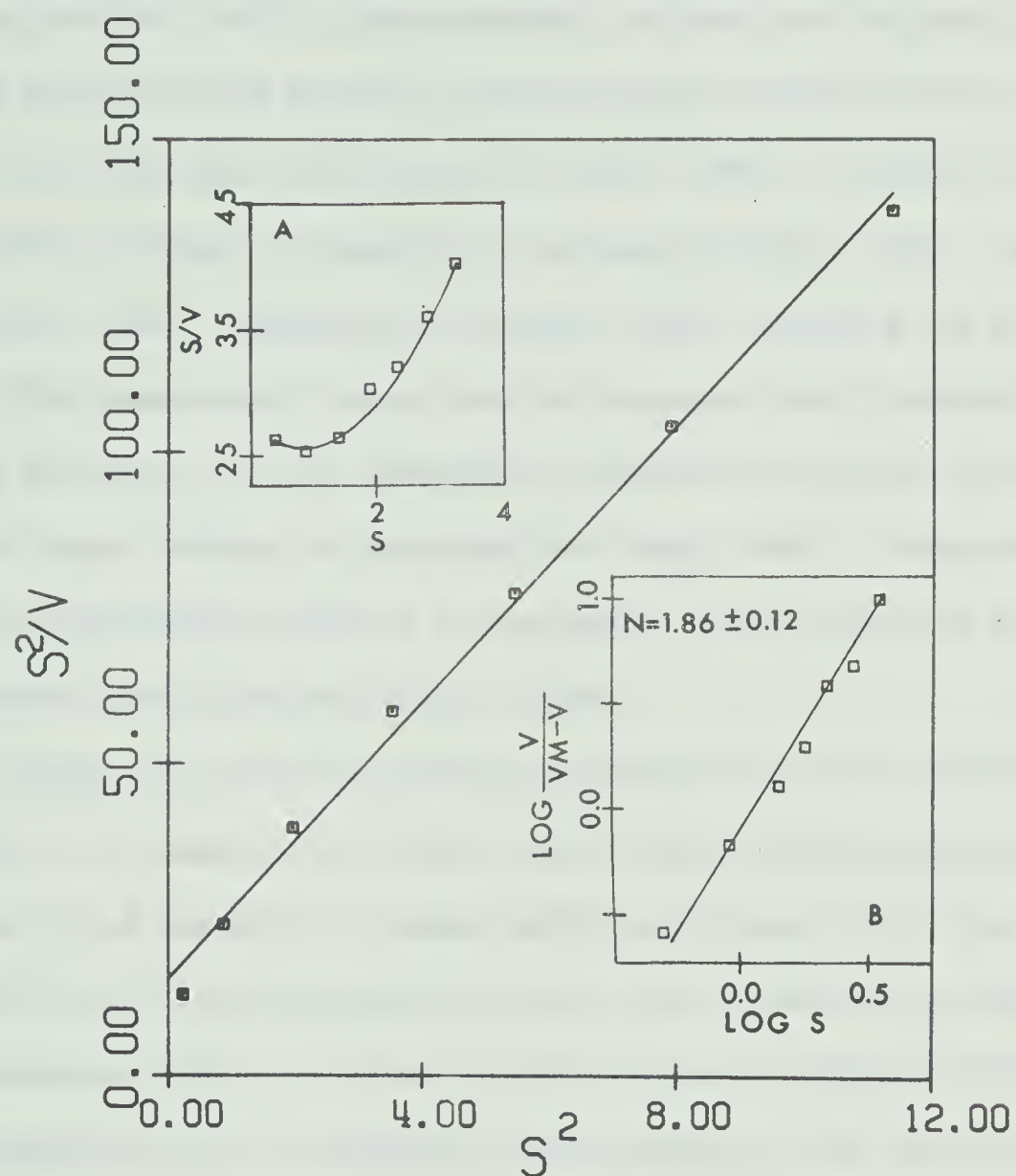


Fig. 44. Effect of DHAP concentrations on housefly TPI activity by the modified assay method. Temp. 30, pH 8.8, 6 ng TPI, $V = \Delta A_{340}/\text{min.}$

cycle intermediates and showed oxidative phosphorylation (Gonda, Traub and Avi-Dor, 1957). Mitochondria isolated from housefly thoraces oxidized glycerol-P and pyruvate, the oxidation of the last was largely controlled by ADP (Van den Bergh and Slater, 1962). Glycerol-P was most readily utilized by housefly mitochondria (Birt, 1961; Chance and Sacktor, 1958; Sacktor and Cochran, 1958; Hansford and Chappel, 1967). High respiratory rates could be obtained with glycerol-P, malate + pyruvate, and the endogenous substrates of intact mitochondria from the locust (Bücher, Klingenberg and Zebe, 1958). Glycerol-P acted as a homotropic effector in the oxidation of glycerol-P by insect mitochondria (Donnellan and Beechy, 1969).

Glycerol-P is the most important substrate at the beginning of flight; the high rate at which flight muscle oxidizes glycerol-P relative to the oxidation of other substrates supports the idea that glycerol-P is the physiological substrate that activates the respiratory chain (Sacktor, 1965). In view of the allosteric control of glycerol-P on the mosquito TPI, it seems that the endogenous level of glycerol-P in flight muscle is a key factor in affecting the flow or concentration of glycerol-P for the respiratory chain. The inhibitory effect of glycerol-P is a negative feedback, leading to a steady pool of glycerol-P. The flight muscle of the blowfly contained an exceptionally high concentration of glycerol-P, 10 to 100 times those of other phosphorylated glycolytic intermediates (Sacktor and Wormser-Shavit, 1966). In contrast with the finding of a large accumulation of glycerol-P during anaerobic glycolysis in insect muscle *in vivo* (Chefurka, 1958;

Heslop, Price and Ray, 1963; Kubista, 1957), the concentration of glycerol-P remained constant during flight (Sacktor and Wormser-Shavit, 1966). Although G-3-P remained constant during the initiation of flight, there was a sharp but transient increase of DHAP (Sacktor and Wormser-Shavit, 1966). Simultaneously, 3-phosphoglycerate also increased; later, the concentration of DHAP became steady during flight. The glycerol-P produced is able to penetrate the mitochondria, inside which it is converted to DHAP by glycerol-P oxidase. So during flight, only a catalytic amount of DHAP is required; the excess DHAP is metabolized to pyruvate (Sacktor and Wormser-Shavit, 1966). A K_m of about 2 mM glycerol-P was obtained for the housefly mitochondrial glycerol-P oxidase (Estabrook and Sacktor, 1958), compared with a K_m of 1.16 mM G-3-P, and an apparent K_m of 1.27 mM DHAP for the housefly TPI, and an apparent K_m of about 2 mM DHAP for the mosquito TPI found in the present investigation. The size of kinetic constants often indicates the *in vivo* concentrations of reactants (Cleland, 1967).

The proportions of different phosphates in the flight muscle of the housefly are: 37% ATP, 20% glycerol-P, 15% Pi, 10% phosphagen, 8% glucose-6-phosphate, 6% AMP, 1% ADP (Winteringham, Bridges and Hellyer, 1955). Blowfly flight muscle contains: ATP 4.09 mg/g wet muscle, Pi 3.10 mg/g, ADP 2.01 mg/g, 3-phosphoglycerate 1.70 mg/g, glucose-6-phosphate 1.55 mg/g, and AMP 0.45 mg/g (Price and Lewis, 1959). For the cecropia moth, Pi and glycerol-P are the main phosphates (Wyatt, Krope and Carey, 1963). Control of respiration in insects by ADP is not as clear as in vertebrates (Sacktor, 1965).

The occurrence of non-competitive inhibition by Pi at low concentrations of HPO_4^{2-} lends evidence to the existence of an allosteric site in the TPI. At low HPO_4^{2-} concentrations, only the allosteric site is affected. At high HPO_4^{2-} concentrations, competitive inhibition also occurs at the catalytic site. The mixed inhibitory pattern observed for other metabolites is consistent with this hypothesis. Pi has a regulatory role in anaerobic glycolysis in the cockroach muscle (Kubista and Foustka, 1962), but is not a rate-limiting factor for metabolism in resting muscle (Nofmanova *et al.*, 1967).

At the onset of flight, the concentrations of ATP and arginine-phosphate decreased, whereas those of ADP, Pi and AMP increased (Sacktor and Hurlbut, 1966). The control of insect TPI by ATP, ADP, GTP, and other nucleotides is probably not as great as that by Pi or glycero-P, as shown by the results (Tables 5, 6). But the summation and the differential of the effects of all these phosphates may contribute to the oscillatory manner of the levels of metabolites in flight muscle as observed by Sacktor and Wormser-Shavit (1966).

The ability of 3-phosphoglycerate, and the Krebs cycle intermediates, citrate, succinate and oxaloacetate to inhibit the mosquito TPI is also a form of negative feedback. By this modulation, glycolytic intermediates can be channeled toward the formation of triglycerides or diglycerides when not demanded for rapid energy production. After incubating rat heart mitochondria for a period of time, citrate, α -ketoglutarate and succinate did migrate and accumulate in the extramitochondrial space (LaNoue, Nicklas and Williamson, 1970). It

was postulated that citrate accumulation, when NADH levels are very high, may turn off the accumulation of NADH in the cytoplasm by inhibiting glycolysis. This observation and hypothesis fits well with the feed-back inhibition of insect TPI by Krebs cycle intermediates. Though an inhibitor for phosphofructokinase from other organisms, citrate had no effect on the locust phosphofructokinase (Walker and Bailey, 1969). The accumulation of citrate in *Tenebrio molitor* pupal tissues suggests the slow utilization of it by the Krebs cycle when least oxygen is consumed (Patterson, 1956). The haemolymph of the larvae of the bot fly, *Gastrophilus intestinalis*, has a citrate concentration 30 times that found in human blood (Levenbook, 1950). Citric acid increased significantly in the housefly after the first day of emergence, but not in *Aedes aegypti* (Zahavi and Tahori, 1965). Citrate is the most abundant intermediate found in mitochondria from both invertebrates and vertebrates (Bellamy, 1961).

The inhibition of mosquito TPI activity by ribose-5-phosphate and folic acid shows that the pentose shunt may be mediated by TPI too. About 17% of the glucose metabolized by the blowfly was via the pentose shunt (Chefurka, 1966).

Newsholme and Taylor (1969) demonstrated that the levels of glycerokinase in insects, whether they use fat or carbohydrates for flight, are 10 to 100 times higher than those in vertebrates. They pointed out the possible conversion of triglycerides to carbohydrates via this enzyme for flight because of the predominance of high rate of glycolysis in insect flight muscle. TPI therefore

occupies a position common to the flow of important metabolites in insects whether they use fat or carbohydrates for flight. For *Prodenia eridania*, a moth which uses fat for flight, the role of carbohydrate metabolism is probably to convert ingested carbohydrates to fat for storage (Stevenson, 1968). The concentration of lipids, mainly diglycerides, in the haemolymph of the desert locust increased about 3 times above the resting level during the first hour of flight (Mayer and Candy, 1969).

In the American cockroach, the glycerokinase activity in leg muscle is about twice that in flight muscle (Newsholme and Taylor, 1969). The level of TPI activity in leg muscle of the cockroach is also about twice that of thoracic muscle (Table 9). These results show that the level of TPI activity is in accordance with the metabolic demand of each particular tissue in co-ordination with other enzymes. Such co-ordination at the cellular level was shown by Pette, Luh and Bücher (1962). During diapause, the cecropia moth accumulated large amount of glycerol-P, which was presumably converted to glycerol (Wyatt and Myer, 1959). The feedback inhibition of insect TPI by glycerol-P could aid the formation of glycerol for diapause.

For flying insects, the thoracic temperature is between 30 and 40 C during flight (Sotavalta, 1954), and the Q_{10} values are: 1.3 to 1.8 for oxygen consumption, 1.3 for wing-beat frequency, and 1.6 for sucrose utilization (Yurkiewicz and Smyth, 1966). These physiological phenomena most likely occur at pace with molecular events. For example, the mosquito TPI activity is linear from 25

Table 9

Levels of α -glycerophosphate dehydrogenase (α -GPDH), triosephosphate isomerase (TPI), and lactic acid dehydrogenase M type (LDH_m) from the crude homogenates of leg muscle, and thoracic muscle of the American cockroach, *Periplaneta americana* (L.)

Enzyme	Leg muscle (units)	Thoracic muscle (units)
α -GPDH	0.540	0.434
TPI	16.200	9.830
LDH _m	0.002	0.001

α -GPDH assayed according to the method of Marquardt and Brosemer (1966): 0.5 mM DHAP, 50 mM Tris, 5 mM EDTA, 0.18 mM NADH pH 6.6. TPI assayed with 1.2 mM G-3-P; the rest as described in methods. LDH_m assayed according to the method of Wilson, Cahn and Kaplan (1963): 0.33 mM pyruvate, 50 mM Tris, 5 mM EDTA, 0.18 mM NADH pH 7.5.

to 40 C, and the Q_{10} is 1.7 (Fig. 7). α -Glycerophosphate dehydrogenase from thoracic muscle of the honey bee also has a Q_{10} of 1.7, and an activation energy of about 10 kcal/mole (Brosemer and Marquardt, 1966). The mechanochemical coefficient of insect flight muscle is 1.0 to 2.4 kcal/mole (Rüegg and Tregear, 1966), compared to an activation energy of 9.94 kcal/mole for mosquito TPI. When an insect is operating at its optimum conditions, the cellular control of TPI by Pi is probably more. This is supported by the inhibition experiments with Pi at different temperatures (Figs. 34, 38, 39). The mosquito TPI is then a system in which the control by Pi is temperature dependent, but the apparent K_m is temperature independent. This is different from the pyruvate kinase system found for fishes: temperature dependent K_m modifications, and temperature independent enzyme-modulator interactions (Somero and Hochachka, 1968). Insect glyceraldehyde-3-phosphate dehydrogenase has evolved a low-temperature capability to retain catalytic power under conditions that will inactivate the same enzyme from other organisms (Gelb and Nordin, 1969).

Substrate inhibition of insect TPI by G-3-P can be interpreted as a form of self-regulating mechanism, in spite of the thermodynamic preference of DHAP as product. It checks the excess flowing of G-3-P toward the glycerol-P shunt. The allosteric nature of insect TPI fits well with the hypothesis that respiratory control in flying insects is not only through the phosphate acceptor level, but also at the substrate level (Chance and Sacktor, 1958). Both Chefurka (1965) and Sacktor (1965) postulated a compartmentalized respiratory

control system in which the concentrations and metabolisms of crucial substrates, cofactors, and precursors are regulated at several points, particularly at branching points of metabolic routes. And this control system can be achieved by feedback mechanisms, or by the competition of several enzymes for a common coenzyme or substrate. Insect TPI plays a role in this system, indeed. It is now pertinent to see if insect hormones can influence the activity of insect TPI, similar to the hormonal control of insect phosphorylase system (Wiens and Gilbert, 1967).

Kinetic Properties of Rabbit TPI

G-3-P to DHAP

With G-3-P as substrate, the Michaelis curve was hyperbolic, and substrate inhibition occurred also (Fig. 45). The K_m was 0.56 mM DHAP by Wilkinson's plot, with 95% confidence limits from 0.38 mM to 0.75 mM. By Lineweaver-Burk plot, the K_m was 0.51 mM; 95% confidence limits from 0.39 to 0.65 mM. An allosteric site was suggested for substrate inhibition of rabbit TPI (Snyder and Lee, 1966). Hill plot of the data prior to substrate inhibition gave an N of 1 (Fig. 45A). The reported K_m values were: 0.46 mM for rabbit muscle TPI (Burton and Waley, 1968a), 0.39 mM for calf muscle TPI (Meyer-Arendt, Beisenherz and Bücher, 1953), 0.35 mM for erythrocyte TPI (Schneider *et al.*, 1965), 0.5 mM for bovine lens TPI (Burton and Waley, 1968b), 0.31 mM for chicken muscle TPI, and 0.75 mM for the rabbit muscle TPI (Wolfenden, 1969). Computer simulation of beef

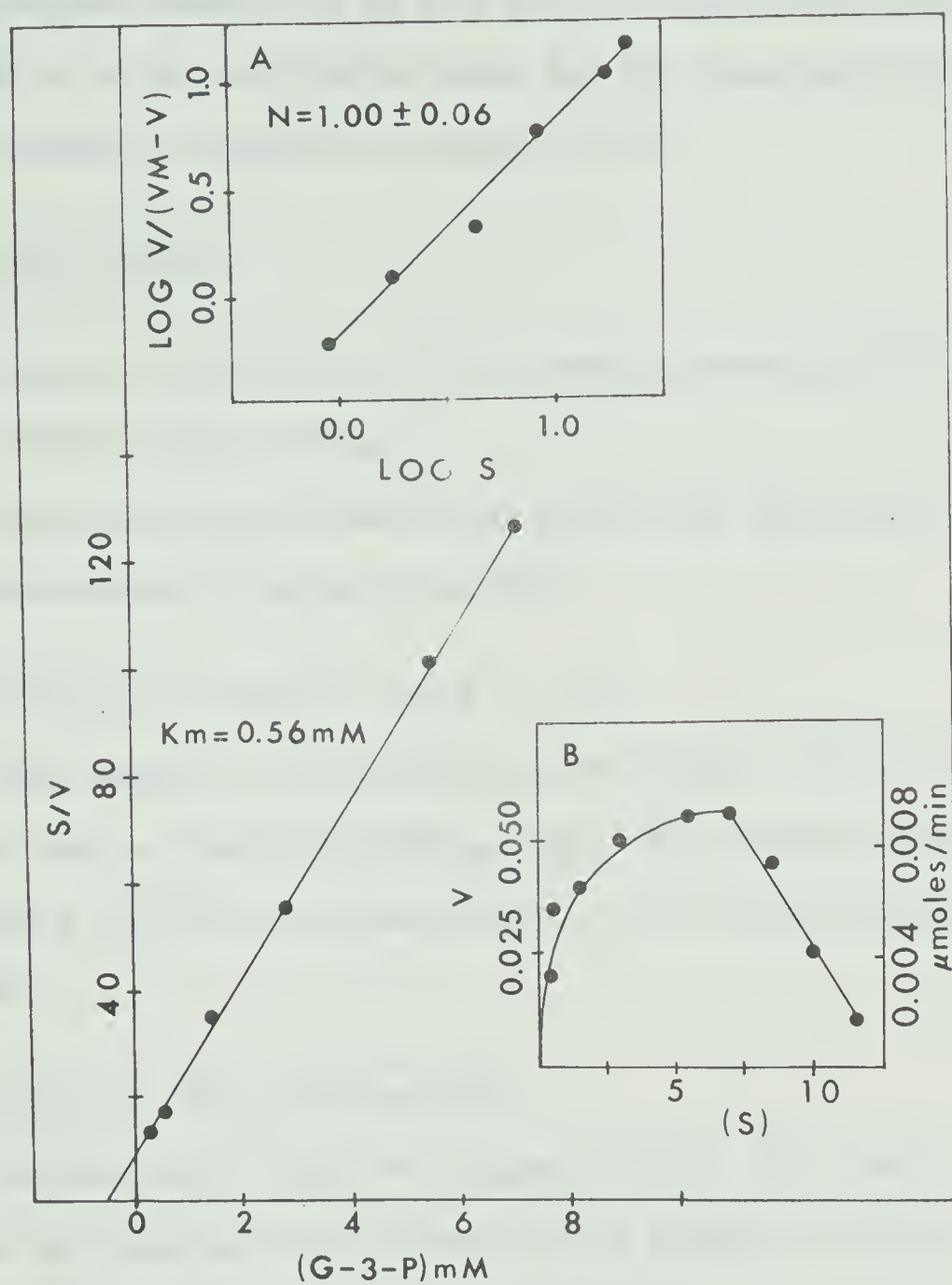


Fig. 45. Effect of G-3-P concentrations on rabbit TPI activity. 1 ng TPI, 50 mM TEA, 5 mM EDTA, pH 8.8, temp. 30 C.
 $V = \Delta A_{340}/\text{min.}$

heart glycolysis showed a K_m of 0.75 mM in an oscillation model, and 0.4 mM in a non-oscillation model for TPI (Achs and Garfinkel, 1968; Garfinkel, Frenkel and Garfinkel, 1968).

DHAP to G-3-P

Conversion of DHAP to G-3-P at varying amounts of TPI (modified assay method)

The reaction was linear up to about 22 ng TPI (Fig. 46). It became zero-order at about 44 ng TPI.

Conversion of DHAP to G-3-P vs. time

When assayed by the coupling enzyme method, the reaction was linear over a 7-min period (Fig. 47A). The reaction rates were curved over a 16-min period when assayed by the modified method (Fig. 47B).

Effect of DHAP concentrations

The Michaelis curves were sigmoidal (Fig. 48A), and non-linearity was observed when S/V was plotted against S (Fig. 49). The kinetic behavior of rabbit TPI could also be approximated by plotting S^2/V against S^2 (Fig. 49B). The apparent K_m was 0.71 mM for the modified method, with confidence limits from 0.53 to 0.87 mM. This apparent K_m value is close to the TPI K_m values found by computer simulation of beef heart glycolysis: 0.53 mM DHAP in a non-oscillating model, and 1 mM in a non-oscillating model (Achs and Garfinkel, 1968; Garfinkel, Frenkel and Garfinkel, 1968).

Determined by the coupling enzyme method, the apparent K_m was 0.95 mM; confidence limits from 0.48 mM to 1.39 mM. Hill plot showed that N was between 2.26 and 1.82 (Fig. 49C).

The Haldane relationship, K_{eq} , was equal to 19 when the maximal velocity obtained by the modified assay method was used. But K_{eq} equaled 27 if the maximal velocity determined by the coupling enzyme method was used. These two values agree well with the reported estimates for the equilibrium constant, 20-28 (Beisenherz, 1955; Lowry and Passonneau, 1964; Meyerhof and Junocwicz-Kocholaty, 1943; Veech *et al.*, 1969).

Inhibitors

All the inhibition experiments were done with the modified assay method.

Some of the mixed inhibitors for the mosquito TPI became non-competitive inhibitors for the rabbit TPI. The fact that the apparent K_m for the rabbit TPI is about 3 times smaller than that of the mosquito TPI may explain this difference in the inhibitory patterns. Folic acid and α -glycero-P were mixed inhibitors (Fig. 50). Folic acid was also probably the most effective inhibitor for the rabbit TPI. Oxaloacetate and succinate were non-competitive inhibitors (Fig. 51). Competitive inhibition was observed at 10 mM citrate, but mixed inhibition at 100 mM citrate (Fig. 52). Both ADP and ATP were non-competitive inhibitors (Fig. 53). The effects of these inhibitors are summarized in Table 10.

The inhibitory effect of IAP (1-hydroxy-3-iodo-2-propanone

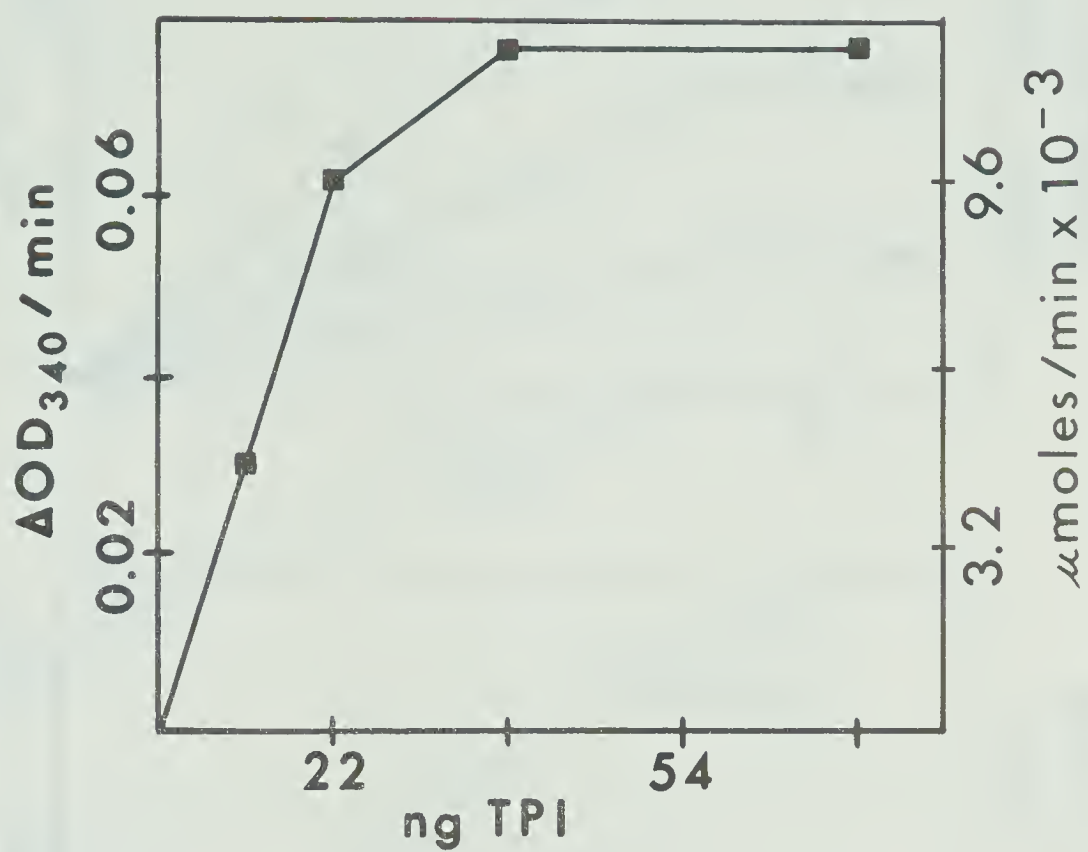


Fig. 46. Conversion of DHAP (1.2 mM) to G-3-P at varying amounts of rabbit TPI. Modified assay method.

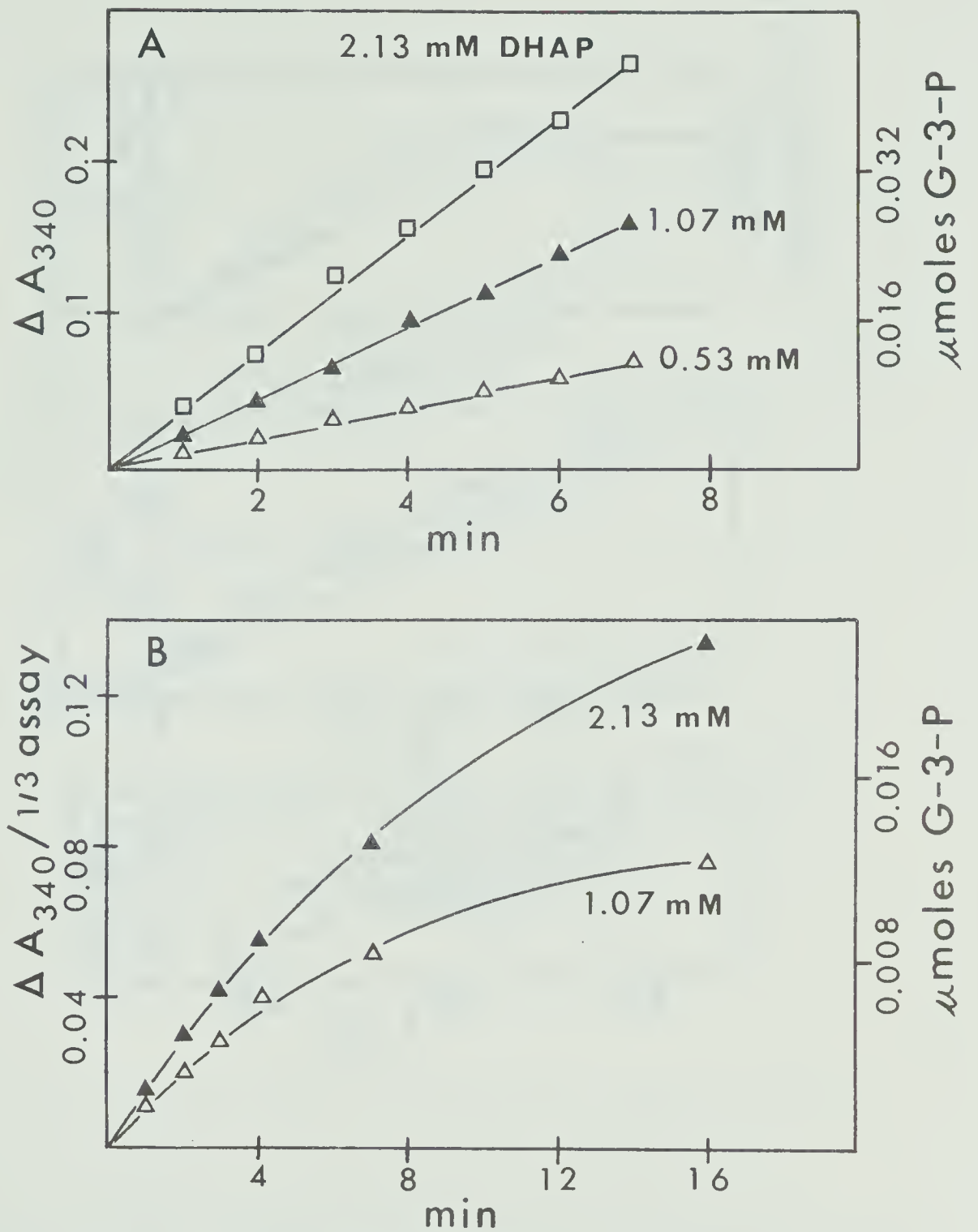


Fig. 47. A. Conversion of DHAP to G-3-P by rabbit TPI vs. time by the coupling enzyme method (18 ng TPI). B. Conversion of DHAP to G-3-P by rabbit TPI vs. time by the modified assay method (11 ng TPI). Temp. 30 C, and pH 8.8 for both assay methods.

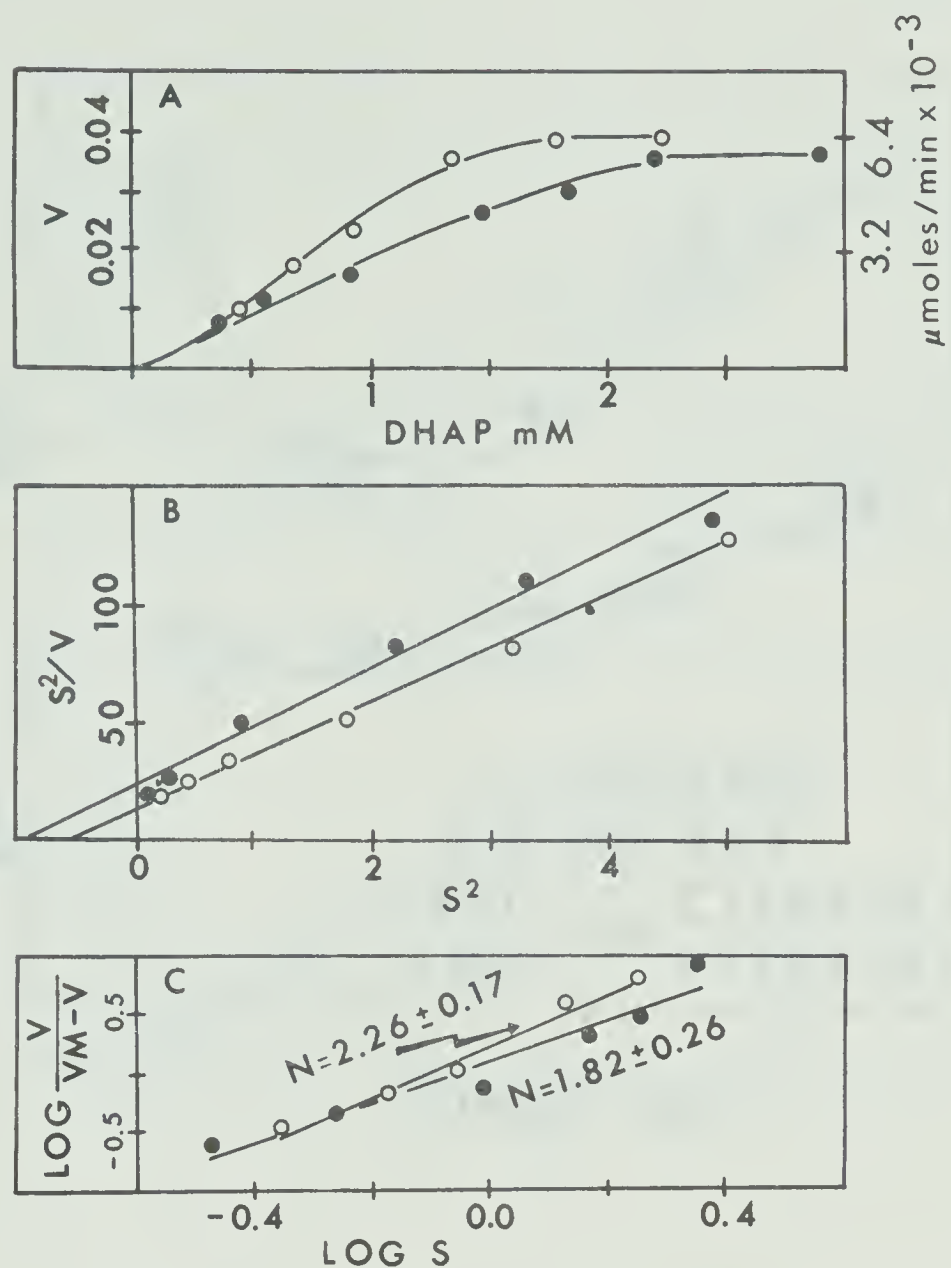


Fig. 48. Effect of DHAP concentrations on rabbit TPI activity.

Open circle for modified assay method; dark circle for coupling enzyme method. 11 ng TPI, temp. 30 C, pH 8.8. $V = \Delta A_{340}/\text{min}$.

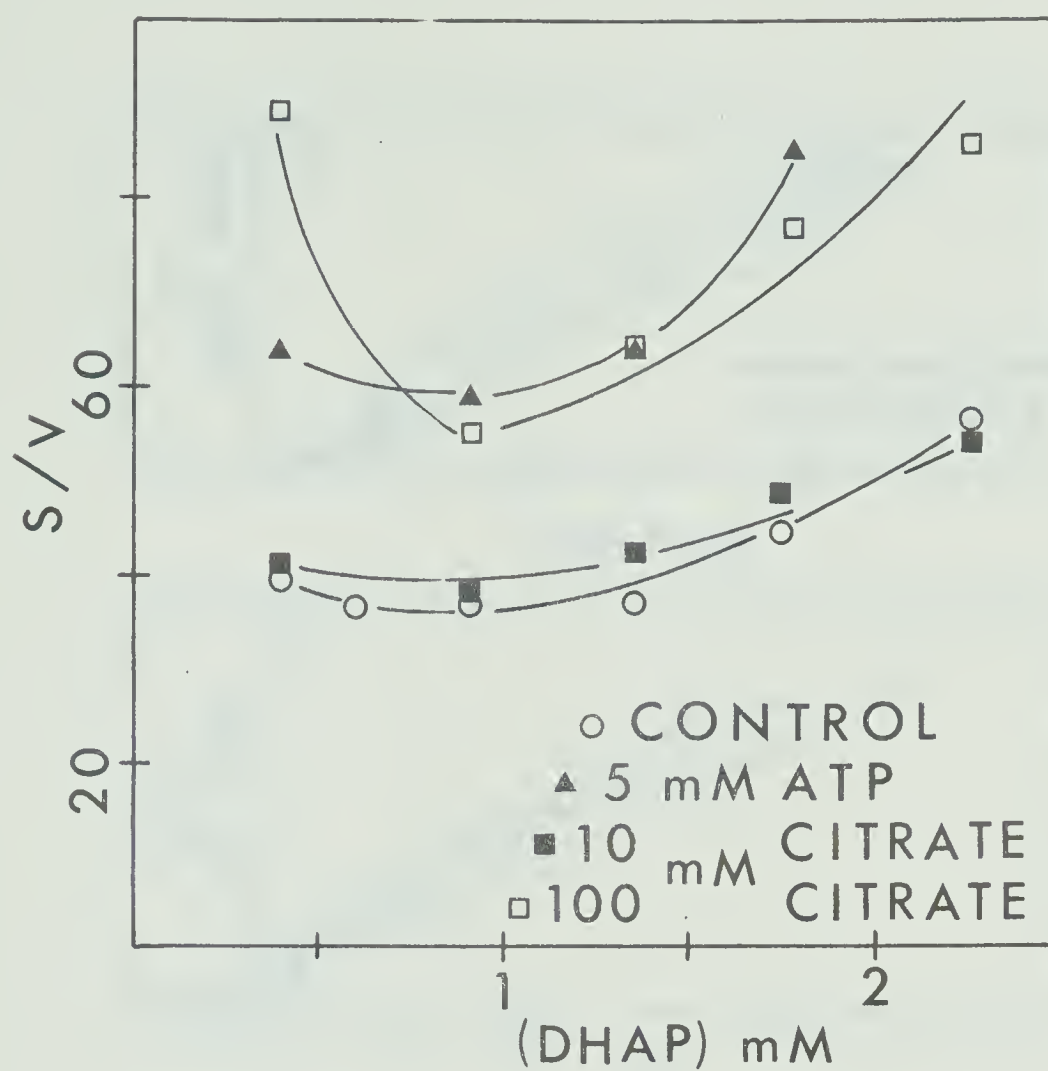


Fig. 49. Wilkinson's plot of kinetic data (DHAP to G-3-P) for rabbit TPI according to the equation: $S/V = K_m/V_m + S/V_m$. The same data also appear in Figs. 49, 52 and 53.

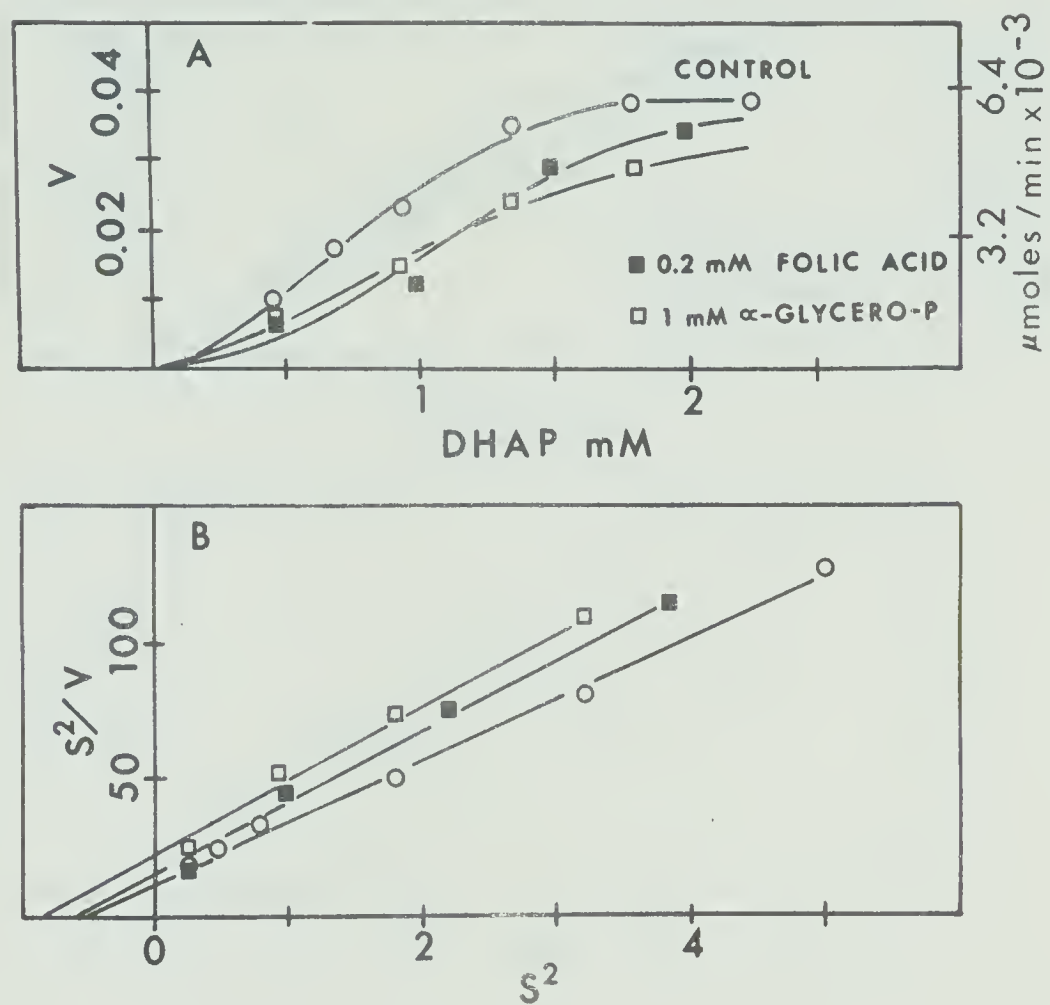


Fig. 50. Effect of folic acid and α -glycerophosphate on rabbit TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

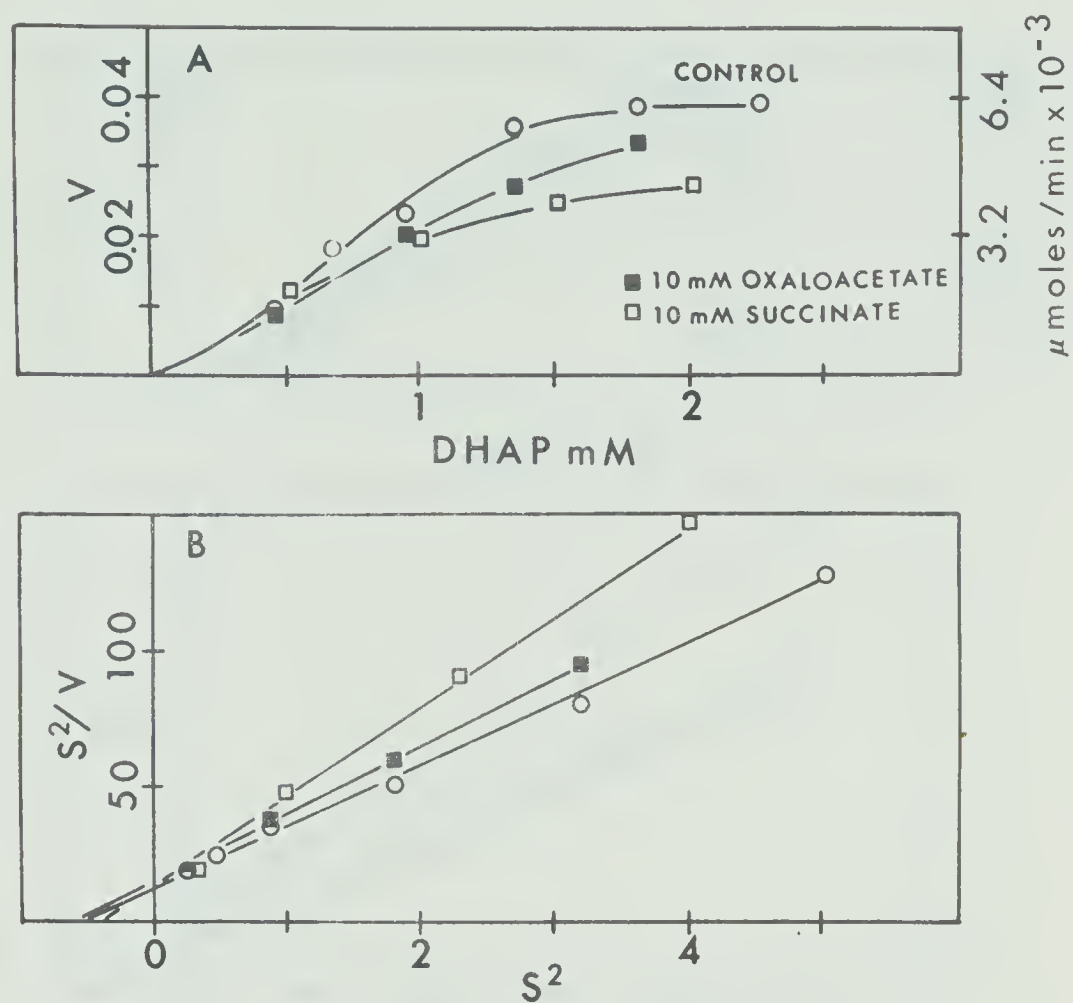


Fig. 51. Effect of oxaloacetate and succinate on rabbit TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

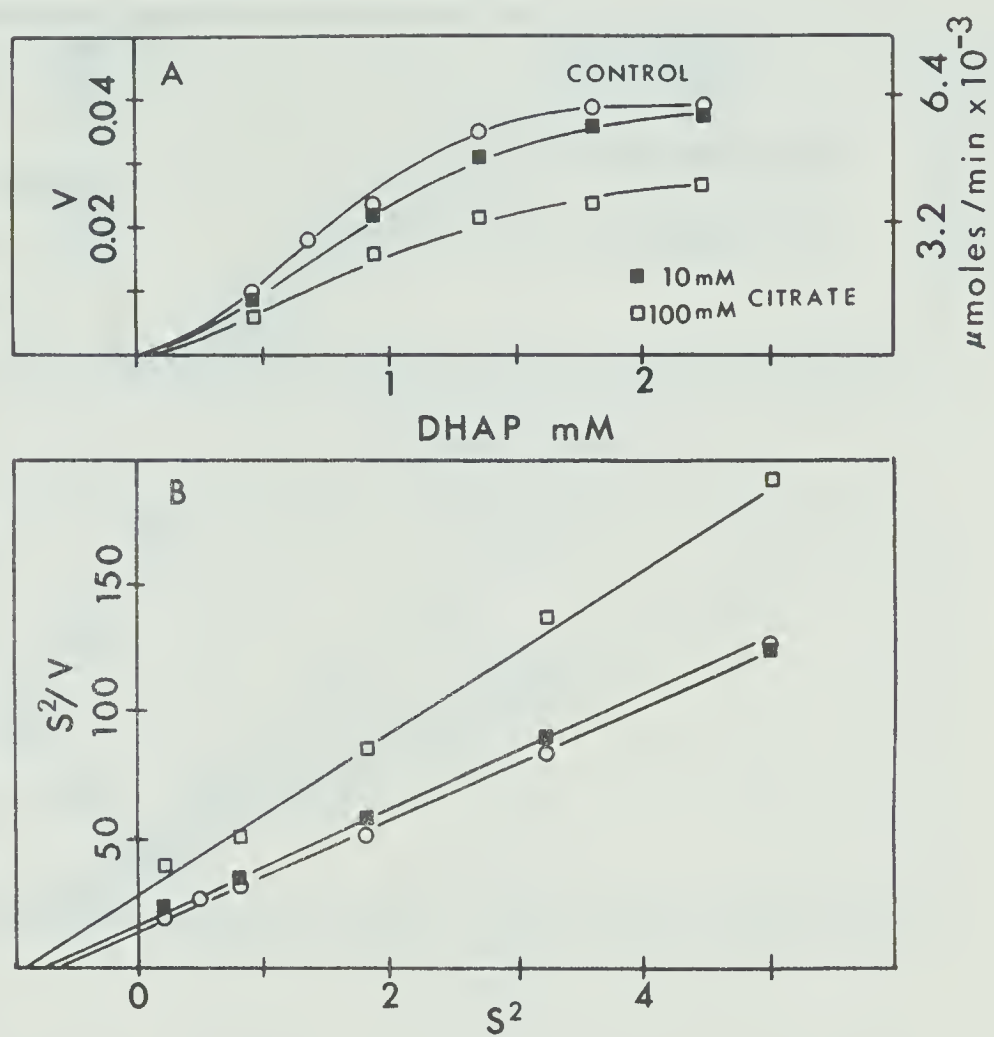


Fig. 52. Effect of citrate on rabbit TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

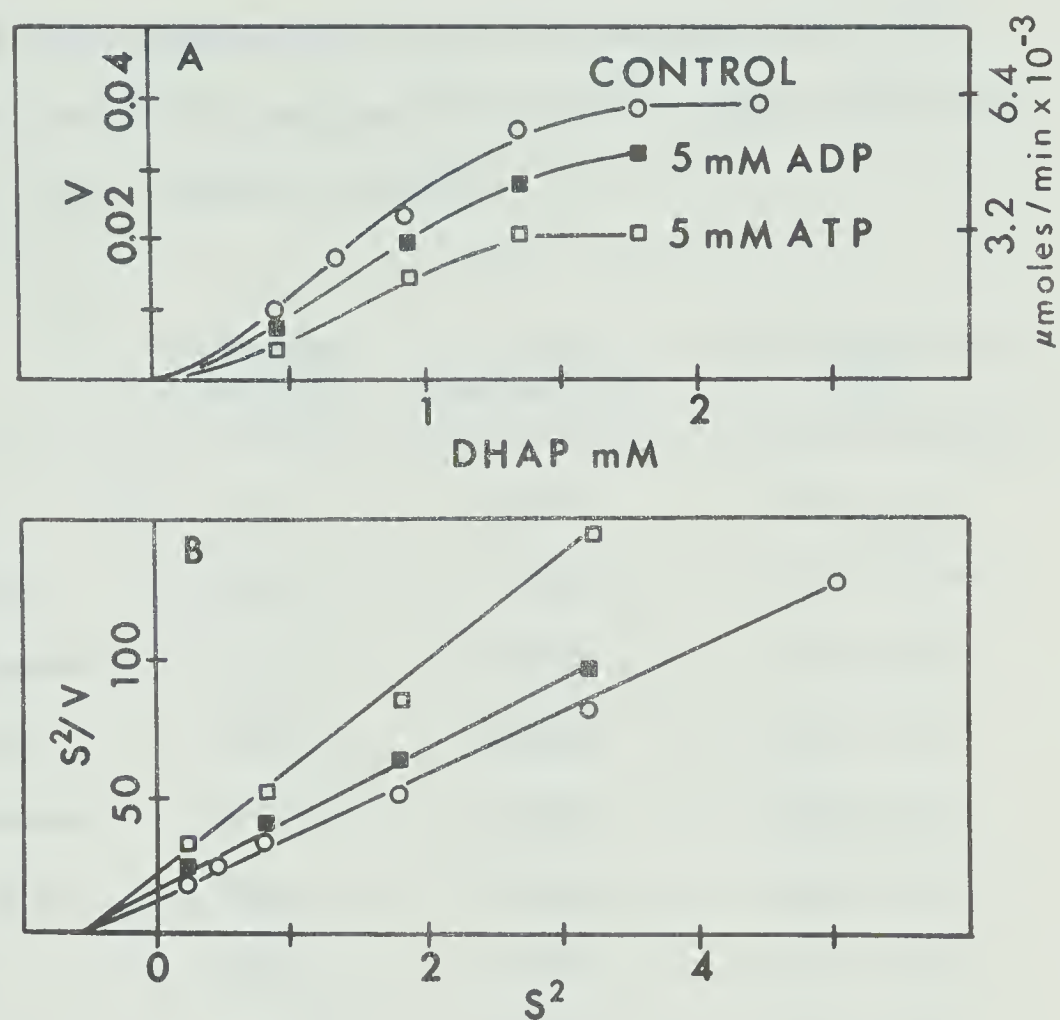


Fig. 53. Effect of ADP and ATP on rabbit TPI activity.

Modified assay method; $V = \Delta A_{340}/\text{min}$.

Table 10

Summary of the estimation of kinetic parameters for rabbit TPI; apparent K_m , and V_m by plotting S^2/V vs. S^2 ; Hill coefficients by the Hill plot. Data from Figs. 49-53.

Experiment	Apparent K_m (mM)	V_m (μ moles/min)	Hill coefficient (N)
Control	0.71	0.0072	2.26 ± 0.17
13 mM Arsenate*	0.95	0.0064	1.82 ± 0.26
1 mM DL- α -Glycero-P	0.92	0.0058	1.74 ± 0.21
0.2 mM Folic acid	0.78	0.0062	1.85 ± 0.20
10 mM Oxaloacetate	0.75	0.0061	1.85 ± 0.12
10 mM Succinate	0.61	0.0046	2.00 ± 0.18
10 mM Citrate	0.89	0.0074	1.94 ± 0.16
100 mM Citrate	0.94	0.0048	2.04 ± 0.05
5 mM ADP	0.76	0.0061	1.88 ± 0.26
5 mM ATP	0.68	0.0040	2.33 ± 0.20

* Coupling enzyme method.

phosphate) was similar to that found for the mosquito TPI (Fig. 54; Table 11). It too could not be analyzed by the Dixon plot. The extent of TPI inhibition by IAP increased as DHAP concentrations were increased from 0.56 to 0.76 mM; further increase in DHAP concentration to 1.12 or 1.50 mM resulted in a marked decline in IAP inhibition of the TPI. This experiment supports the claim that cooperativity and conformational change might be involved.

By the Dixon plot, α -glycero-P was probably a mixed inhibitor, with a K_i of 0.96 mM (Fig. 55). With G-3-P as substrate, Wolfenden (1969) reported a K_i of 0.23 mM DL- α -glycero-P for rabbit TPI. A glycolytic intermediate, 3-phosphoglycerate, was also an inhibitor, giving a K_i of 25.55 mM (Fig. 56). Ribose-5-phosphate, a pentose shunt intermediate, was less effective than 3-phosphoglycerate, with a K_i of 53.88 mM (Fig. 57). N was 1.71 at 0.96 mM DHAP, but became 0.94 at 1.57 mM DHAP (Fig. 57A). P_i was a mixed inhibitor; K_i was 1.29 mM DHAP (Fig. 58). The inhibition was non-linear at 1.05 mM DHAP, with saturation 4 mM P_i . N was 1.75 at 1.05 mM DHAP, and 1.05 at 1.41 mM DHAP (Fig. 47A). With G-3-P as substrate, a K_i of 4.8 mM P_i was observed (Wolfenden, 1969).

Equilibrium dialysis with radioactive P_i

So far, the experiments with various ligands indicated that there are 2 binding sites on the TPI moiety. Therefore, equilibrium dialysis was carried out with radioactive P_i to see if the order of binding was the same. A non-linear relationship was observed by

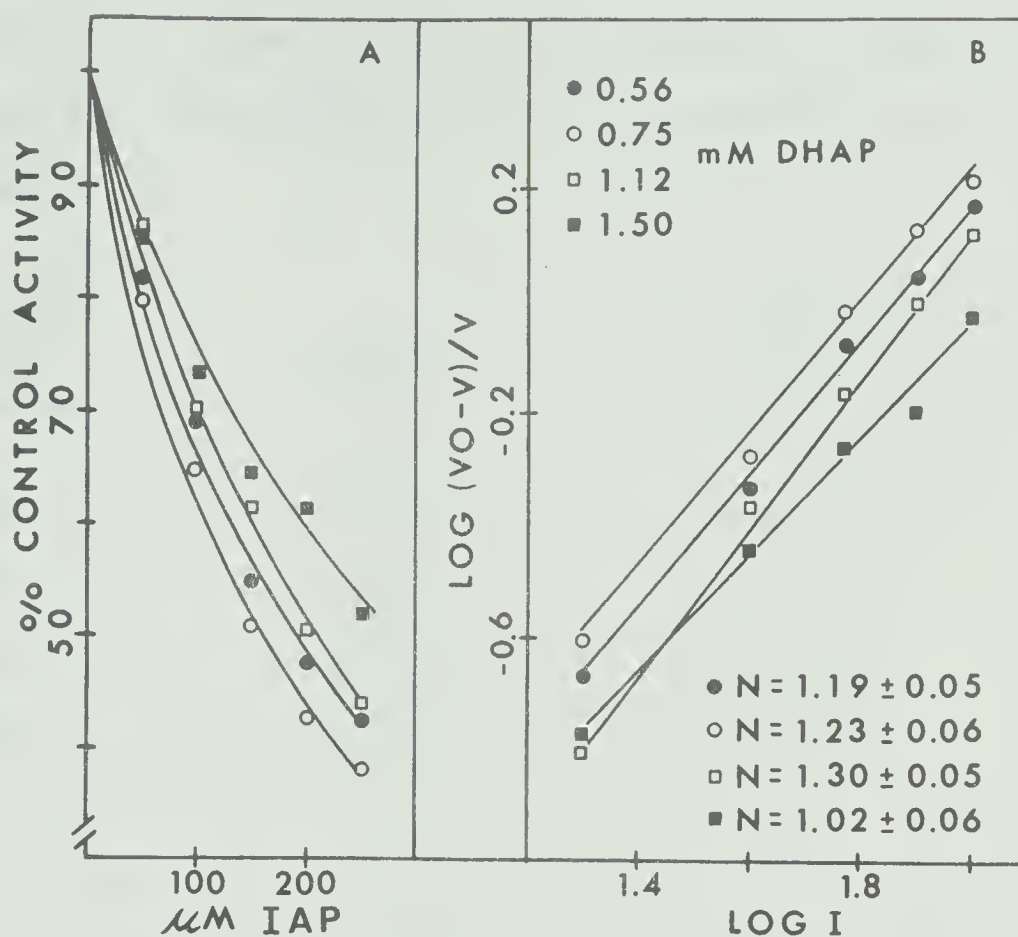


Fig. 54. Effect of 1-hydroxy-3-iodo-2-propanone phosphate (IAP) on the conversion of DHAP to G-3-P by rabbit TPI. Modified assay method; $V = \Delta A_{340}/\text{min}$; V_0 is the velocity in the absence of inhibitor.

Table 11

Summary of the effect of IAP on the conversion of DHAP to G-3-P by rabbit TPI. Data from Fig. 54. I_{50} estimated by Hill plot (Fig. 54B).

DHAP conc. (mM)	I_{50} (μ M)	Hill coefficient (N)
0.56	184.02	1.19 ± 0.05
0.75	158.76	1.23 ± 0.06
1.12	205.43	1.30 ± 0.05
1.50	282.49	1.02 ± 0.06

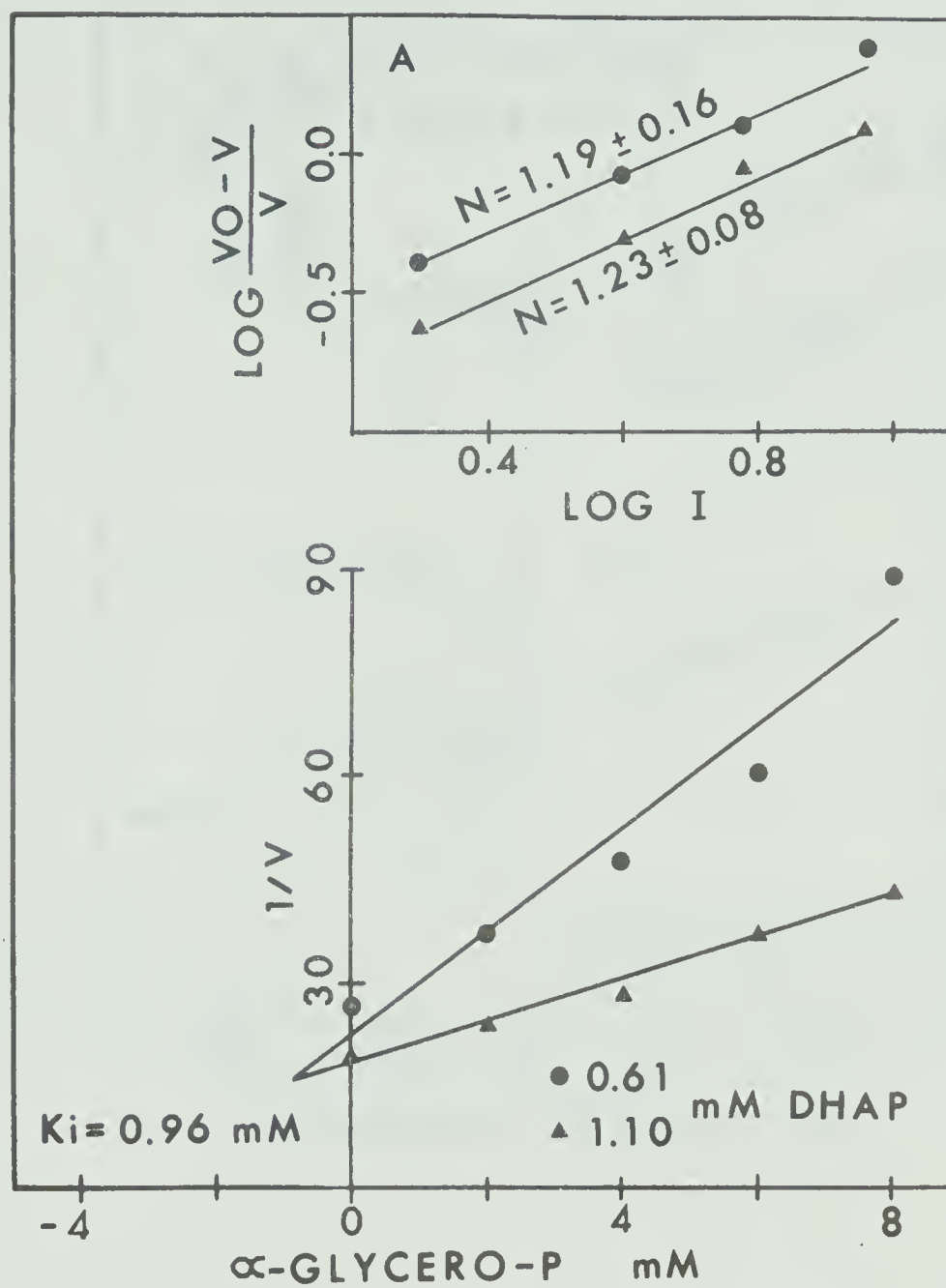


Fig. 55. Effect of DL- α -glycerophosphate on rabbit TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min}$.

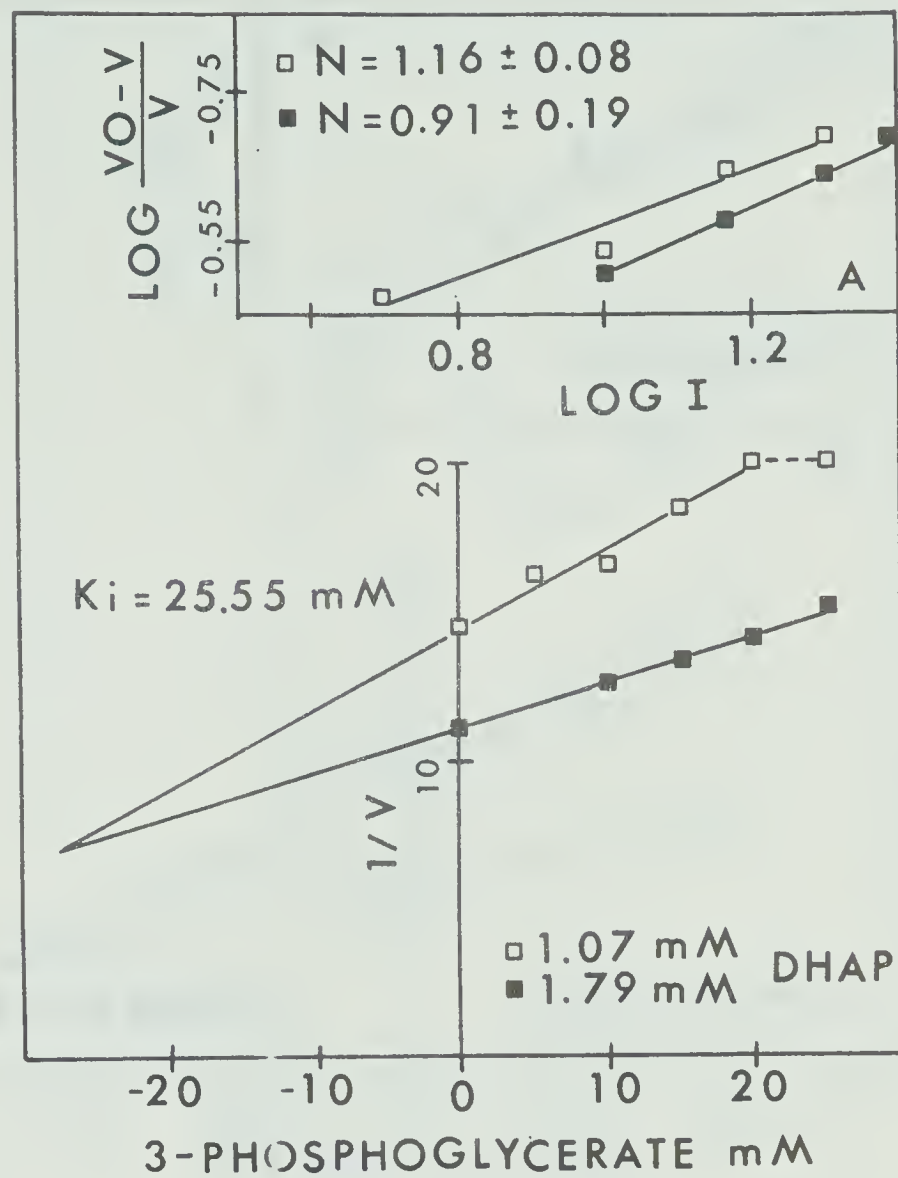


Fig. 56. Effect of 3-phosphoglycerate on rabbit TPI activity.

Modified assay method; $V = \Delta A_{340}/\text{min}$

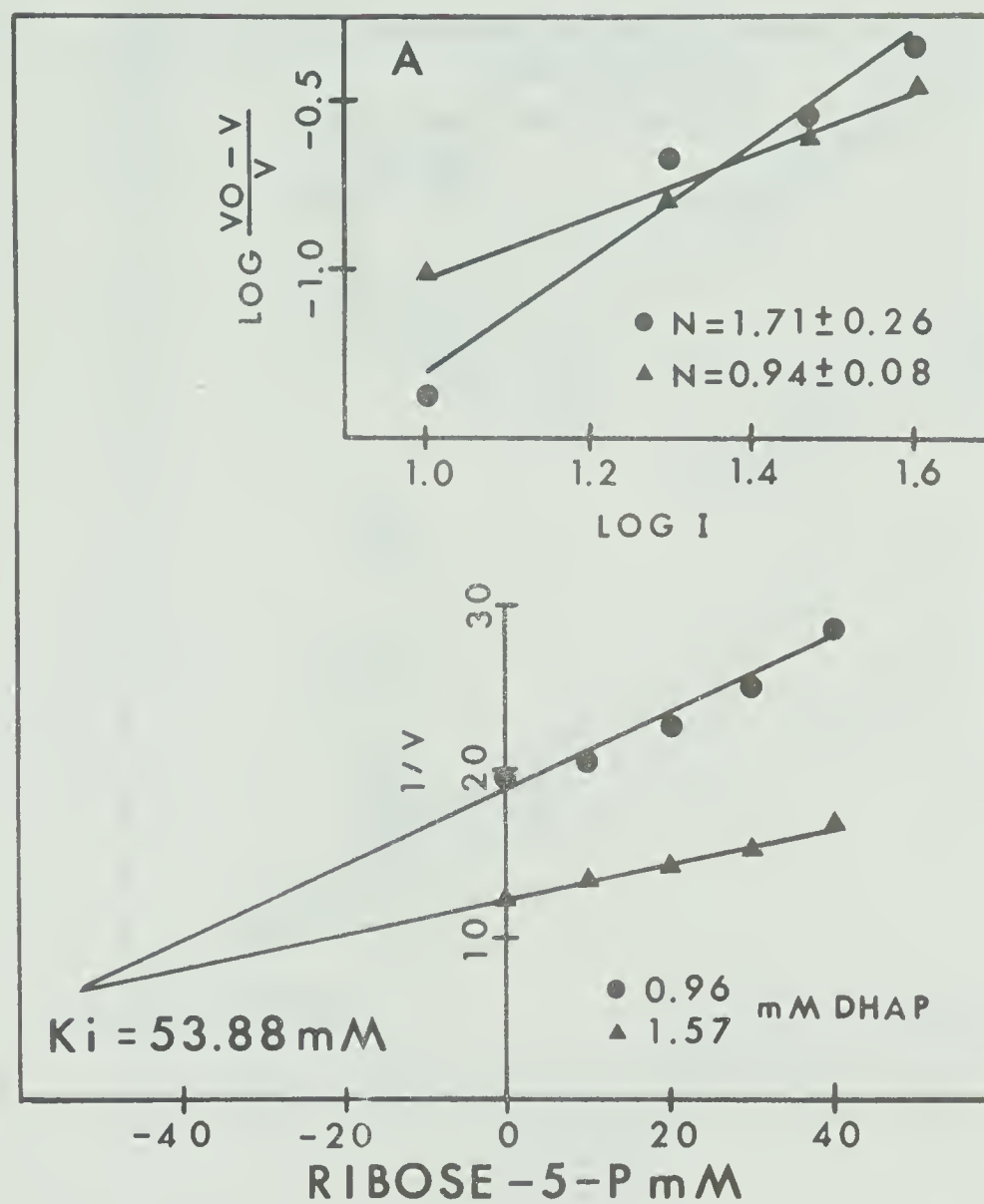


Fig. 57. Effect of ribose-5-phosphate on rabbit TPI activity.
 Modified assay method; $V = \Delta A_{340}/\text{min}$.

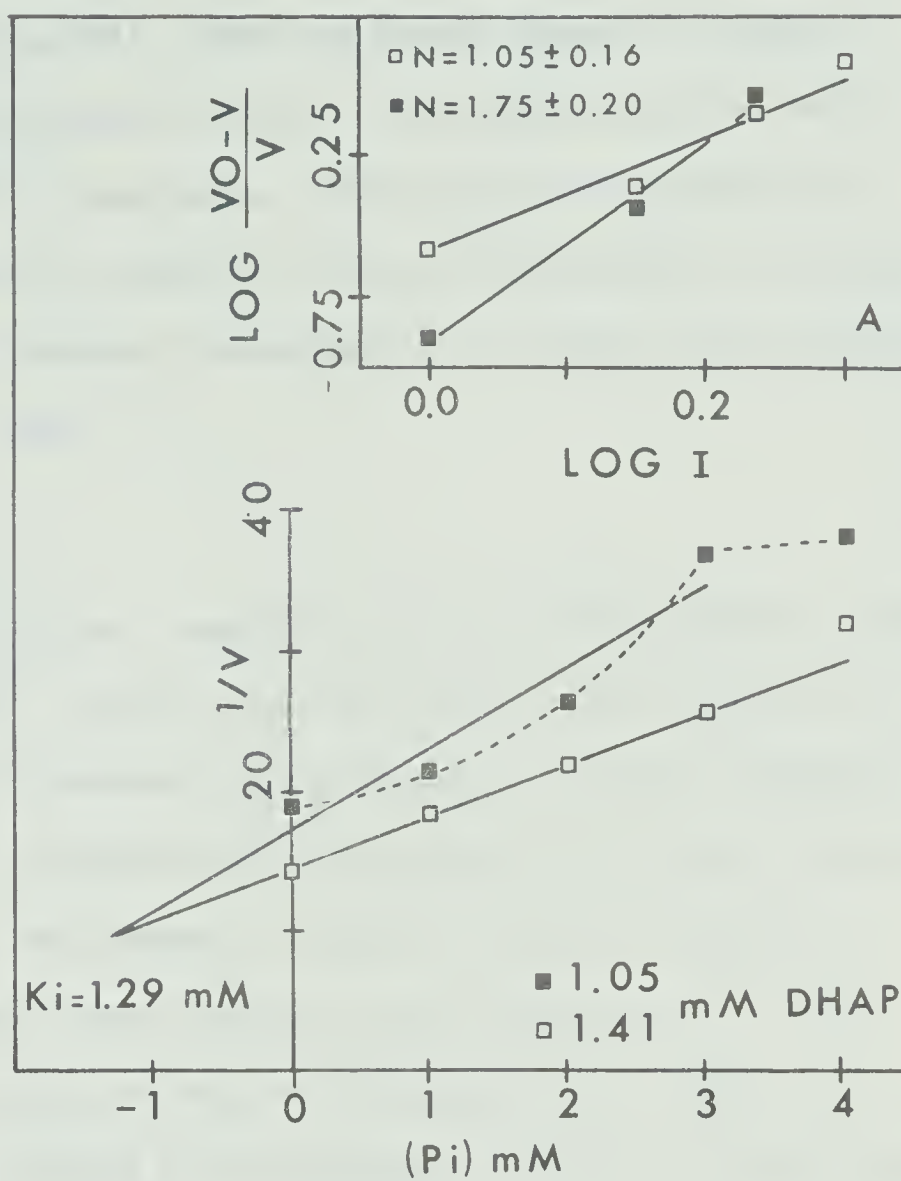


Fig. 58. Effect of Pi on rabbit TPI activity. Modified assay method; $V = \Delta A_{340}/\text{min.}$

the Scatchard plot (Fig. 59). Thus the actual number of binding sites could not be determined directly. The highest binding ratio (r) obtained was 1.44. Nonetheless, this experiment supports the hypothesis that there are probably 2 sites on TPI moiety for binding ligands. Curvature indicates heterogeneity of binding sites (Koshland, Conway and Kirtley, 1968).

Discussion II

Rabbit TPI is also a regulatory enzyme with properties similar to that of insect TPI. But the role TPI plays in the regulation of metabolism in mammals remains to be elaborated. It may be involved in the channeling of carbohydrates into pentoses, glycolysis, glycerides, or more importantly, the gauging of glycerol-P shunt in mammalian tissues. The glycerol-P shunt has been found operative in: liver, heart and skeletal muscle of the rat (Kleitke *et al.*, 1969); rat brain mitochondria (Kleitke and Wollenberger, 1969); and white muscle of the guinea pig (Blanchaer, 1964; Lundquist and Kissling, 1967). White muscle mitochondria had a higher rate of oxygen consumption with glycerol-P than with lactate, or succinate (Blanchaer, 1964). The oxygen consumption rate of white muscle mitochondria was the same either with glycerol-P, or with pyruvate + malate (Lundquist and Kissling, 1967). Changes of glycerol-P concentration in rat adipose tissue is related to the concentration of DHAP in the cytoplasm, but not related directly to alterations in glycolytic rate (Halperin and Denton, 1969). The provision of glycerol-P for glyceride synthesis may be the role of glycolysis in adipose tissue under certain conditions

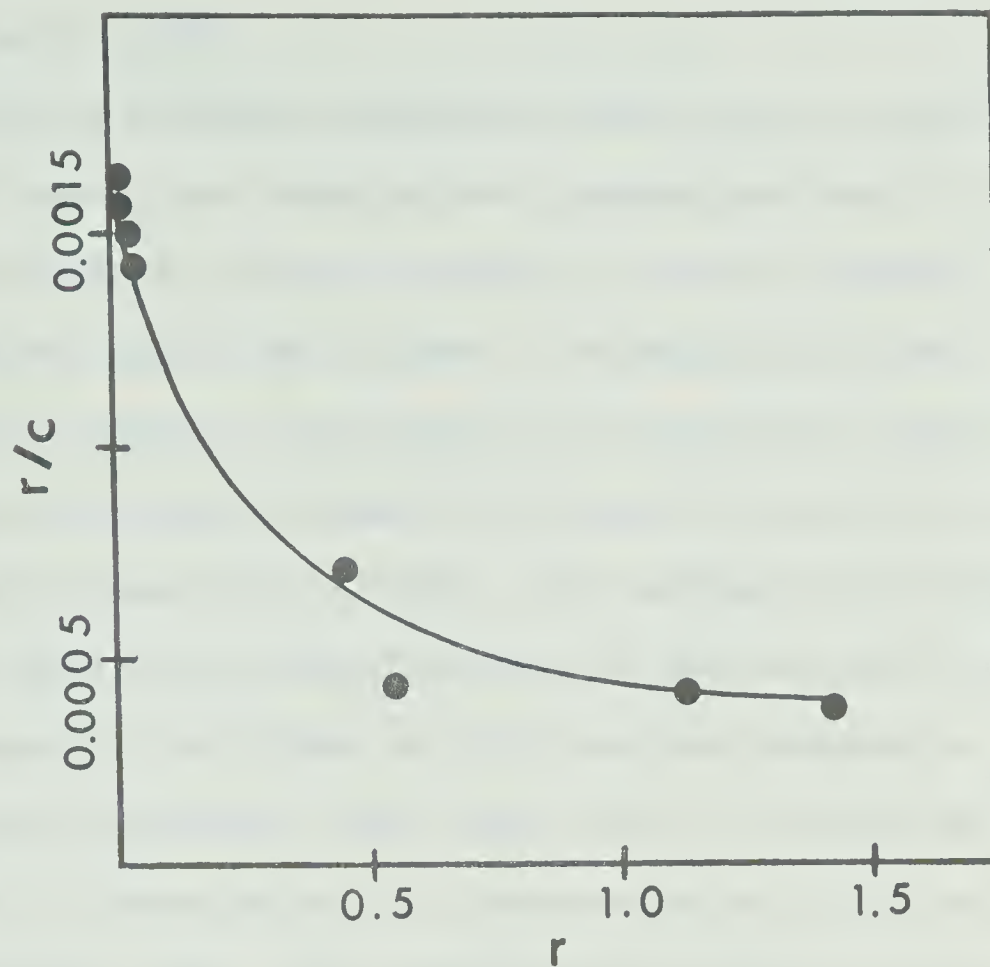


Fig. 59. Scatchard plot of the binding of Pi to rabbit TPI by equilibrium dialysis; r the molar ratio of Pi bound to TPI, and c the unbound Pi concentration.

(Halperin and Denton, 1969).

With G-3-P as substrate, Wolfenden (1969) found that rabbit and chicken TPI activity was inhibited by: phosphoglycollate, Pi, β -hydroxymethyl phosphate, ribose-1-phosphate, glycerol-P, acetate, glycollate, oxalate, malate and succinate. Phosphoglycollate was the most effective inhibitor, and glycerol-P the second most effective inhibitor. The *in vivo* ratio of DHAP/G-3-P was 9.3 in rat liver, and 12.2 in rat muscle (Veech *et al.*, 1969). This confirmed the findings of Rose *et al.* (1962) of the nonequilibration of DHAP and G-3-P in rat liver. Disequilibrium of DHAP and G-3-P was also observed in ascites tumor cells (Garfinkel, 1963; Hess, 1963); G-3-P was 50% higher than DHAP in concentration, and compartmentation effect was suggested (Garfinkel, 1963). This compartmentation effect can now be attributed to the feedback by various metabolites. The inhibition of lactate synthesis in Ehrlich ascites tumor cells incubated with formiminoglutamate was attributed to the inhibition of TPI by a metabolite of formiminoglutamate (Fontenelle and Henderson, 1969). Thus carbohydrate utilization, whether for anabolic or catabolic purpose, can be modulated at the step of TPI.

There are various pathological conditions associated with the activity of TPI. By electrophoresis, TPI-deficient hemolysate revealed a moderate decrease in the activity of the slowest moving band (BB), and the virtual disappearance of the 2 faster moving bands, AA and AB (Kaplan *et al.*, 1968). A deficiency of TPI may cause nonspherocytic hemolytic disease, associated with increased susceptibility to infection, and with neurologic disorders (Schneider *et al.*, 1965).

This rare disorder is inherited by an autosomal gene, and the symptom-free heterozygotes have an intermediate TPI deficiency. TPI activity is lower in Ehrlich ascites tumor cells (Hirsch-Hoffman, Hoezel and Maass, 1964). In mice bearing the Ehrlich carcinoma and thymoma, the TPI activity is increased (Schade, 1953). The elevation of TPI activity to 3 times that of normal is found in generalized neoplastic diseases, such as hepatic metastasis, liver cirrhosis, hepatitis, acute leukemia, pulmonary and myocardial infarcts, and acute hemorrhagic pancreatitis (Robert, Van Rymenant and Lagae, 1961). TPI activity is higher than normal in myelocytic leukemia leucocytes although the glycolytic rate is lower than normal (Beck, 1955).

CONCLUSION AND GENERAL DISCUSSION

Regardless of tissue origin, TPI is a regulatory enzyme possessing an allosteric site. The term allosteric and regulatory are often interchangeable. A regulatory enzyme is defined as one in which the activity is controlled by factors other than substrate availability, and the activity of which controls the rate of flux or the concentrations of metabolic intermediates of a metabolic pathway (Newsholme and Gevers, 1967). Or, an allosteric enzyme has one or more of the following attributes (Koshland and Neet, 1968): (1) contains a site topologically distinct from the active site; (2) shows sigmoidal kinetics; (3) occurs at a branch point in a metabolic pathway; (4) obeys the symmetry or concerted model. Substrate inhibition of an enzyme is indicative of regulatory mechanism (Krebs, 1964).

TPI has the following attributes that qualify it as an allosteric enzyme: (1) TPI shows sigmoidal kinetics. (2) It occurs at a branch point, and is subject to control by metabolites of the pentose shunt, glycerol-P shunt, glycolysis, and Krebs cycle. (3) It is subject to substrate inhibition by G-3-P (Figs. 42, 45). (4) It probably mediates the rate of flux of some metabolites by feedback mechanisms. The results of Fontenelle and Henderson (1969) substantiate this claim. (5) The shift from mixed, or possibly competitive, to non-competitive inhibition observed for the experiments with P_i points to the existence of an allosteric site topologically distinct from the active site (Figs. 33-36, 38, 39). However, it is uncertain whether this allosteric

site can also be functionally catalytic. (6) TPI functions as a dimer, and cooperativity among inhibitors could be observed, i.e., alteration of N values with changing substrate concentrations. When the interaction is very strong, N approaches the number of binding sites for a ligand (Madsen and Shechosky, 1967). (7) TPI could undergo conformational change. This is deduced from the experiments with IAP (Figs. 32, 54). The inactivation of aldolase by IAP was possibly due to induced conformational change (Hartman, 1970b). When crystals of rabbit TPI were soaked in phosphoglycollate, the major units of orthorhombic unit cell contracted 6.1%, and upon the removal of the inhibitor, the crystals expanded to the original dimension (Wolfenden and Johnson, 1970). Glycero-P was less effective, but produced 3.6% contraction along the same axis.

The relationship of TPI with other metabolites is shown by Fig. 60. The observation by LaNoue *et al.* (1970) that citrate migrates out of the mitochondria and accumulates in the cytoplasm can now be viewed in a more appropriate perspective--feedback information. Feedback can lead to both compartmentation and energy production simultaneously. Negative feedback relationships are stabilizing; positive feedback leads to instability, and in some cases oscillation (Atkinson, 1966). Considering the number of metabolites that can inhibit TPI activity, it seems that TPI does not operate at its full capacity *in vivo*. The increase in the total specific activity of mosquito TPI after Sephadex G-50 chromatography supports this hypothesis (Table 2). The unequal labelling of hexoses in tracer

studies was accounted for by assuming that the reaction catalyzed by TPI was slow (Ljungdahl *et al.*, 1961). The disequilibrium between DHAP and G-3-P is "very strange in view of the very large enzymic activity of the isomerase" (Garfinkel *et al.*, 1968). The disequilibrium between DHAP and G-3-P is most likely due to the inhibition of TPI by certain metabolites. The reaction catalyzed by TPI is classified as one that is in quasi-equilibrium, and Pi is an important factor in controlling reactions that are in quasi-equilibrium (Hess, 1963). Control by Pi is the most essential feature in the regulation of metabolism (Racker, 1965; Uyeda and Racker, 1965). Chapman and Bartley (1969) proposed that adenosine phosphates do not control the direction of glycolytic flux, but rather by enzyme activation and inactivation. It is difficult to correlate here the inhibition of TPI by various metabolites with the Pasteur effect, or the Crabtree effect, especially when Pi can stimulate hexokinase and phosphofructokinase in reconstructed systems (Uyeda and Racker, 1965). But perhaps, the metabolites can be assigned a role comparable to that of neuronal noises in neurophysiology (Stein, 1970). Rather than being inhibitors per se, the metabolites may serve to smooth out the reaction catalyzed by TPI, imposing upon it a pacemaker mechanism. If so, then these metabolites act as messengers--information transmission. Information transmission is not the primary aim for the biological organism, but rather for maximum survival (Stein, 1967). Inhibition of an enzyme that is in excess will not produce a marked effect on metabolism until its activity has been reduced to the point where it

is limiting (Webb, 1963).

It is probably premature to speculate on the allosteric mechanism by which TPI operates. It may involve conformational change, as that of Koshland, Némethy and Filmer (1966). Or it can belong to the 'K' system according to the model of Monod *et al.* (1965). The apparent fit by the plotting of S^2/V against S^2 suggests that the reaction can be approximated by eq. 11. But the kinetic behavior of TPI is undoubtedly more complicated than this simple approximation. The findings that there are 2 subunits in TPI (Burton and Waley, 1966; Johnson and Waley, 1967), and that 1.7 moles of IAP were bound per mole of rabbit TPI, plus the results from the present investigation, support the proposal that a second order reaction takes place in either direction.

SUMMARY

1. Triosephosphate isomerase has been purified from the thoracic muscle of the housefly, *Musca domestica*, by ammonium sulfate fractionation, DEAE cellulose, and Sephadex G-100 column chromatography.
2. Triosephosphate isomerase has also been purified from the thoracic muscle of the yellow fever mosquito, *Aedes aegypti*, by acetic acid precipitation, Sephadex G-50, DEAE cellulose, and Sephadex G-100 column chromatography.
3. The estimated molecular weight for triosephosphate isomerase from the housefly, mosquito, and rabbit is 60,000.
4. Both the housefly and rabbit triosephosphate isomerase are electrophoretically cationic.
5. The activity of triosephosphate isomerase from leg muscle of the cockroach, *Periplaneta americana*, is about twice that of thoracic muscle.
6. The pH optimum for the mosquito and housefly triosephosphate isomerase is about pH 8.8.
7. With dihydroxyacetone phosphate as substrate, the triosephosphate isomerase activity shows sigmoidal kinetics, and can be approximated by assuming a second order Michaelis-Menton equation.
8. For the mosquito triosephosphate isomerase, the apparent K_m does not change from 25 C to 40 C. The apparent K_m is

about 2 mM dihydroxyacetone phosphate.

9. For the housefly and the rabbit, the apparent K_m with dihydroxyacetone phosphate as substrate is 1.1 mM, and 0.71 mM respectively.
10. With glyceraldehyde-3-phosphate as substrate, the isomerase activity can be described by the simple Michaelis-Menton equation, except the occurrence of substrate inhibition. The K_m is 1.16 mM for the housefly, and 0.56 mM for the rabbit.
11. Triosephosphate isomerase is subject to inhibition by: inorganic phosphate, DL- α -glycerophosphate, β -glycerophosphate, 3-phosphoglycerate, ribose-5-phosphate, ADP, ATP, CTP, CDP, UTP, GTP, citrate, oxaloacetate, succinate, folic acid, 1-hydroxy-3-iodo-2-propanone phosphate, and arsenate.
12. Conformational change on the enzyme moiety is suggested by the experiments with 1-hydroxy-3-iodo-2-propanone phosphate.
13. The inhibition of mosquito triosephosphate isomerase activity by inorganic phosphate shifted from mixed to non-competitive by lowering the pH from 8.0 to 6.5. The apparent K_i changed from 0.8 mM to 4.2 mM.
14. For the mosquito triosephosphate isomerase, the pattern of inorganic phosphate inhibition shifted from non-competitive at 25 C to mixed at 30 and 40 C. The K_i changed from 4.37 to 0.27 mM; activation energy was +32.69 kcal/mole.
15. Cooperativity among ligands exists when binding onto the

enzyme moiety. There are possibly two sites for binding ligands.

16. Scatchard plot of equilibrium dialysis with radioactive inorganic phosphate was non-linear.
17. The binding of ligands onto the enzyme moiety seems to involve the 2 oxyanions on these ligands.
18. It is postulated that triosephosphate isomerase is a regulatory enzyme with an allosteric site.

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APPENDIX

This appendix contains digital computer programs written in APL\360 for the analysis of kinetic data for triosephosphate isomerase.


```

      ∇ WIN[ ] ∇
      ∇ X WIN V;T;Q;W;X;Y;A;B;KM;L;D;J;O;A;B;C;T2;E;TE
[1]  T←(5 3)ρ0
[2]  'S:                      S/V: ';3 RND Q←Q(2,N←ρX)ρX,(Y←X÷V)
[3]  T←X SR Y
[4]  X←T[1;1]
[5]  KM←(A←T[3;1])÷B←T[4;1]
[6]  D←(+/(Y-(A+B×X))*2)÷J←N-2
[7]  L←D×(1÷N)+(X*2)÷W←+/(X-X)*2
[8]  O←(M←D÷W)×-X
[9]  TE← 0 4.303 3.182 2.776 2.571
      2.447 2.365 2.306 2.262 2.228
[10] A←(B*2)-(T2←TE[J]*2)×M
[11] B←(A×B)-T2×O
[12] C←(A*2)-T2×L
[13] E←((B*2)-A×C)*0.5
[14] 'Y                        = ';3 RND B;'X + ';3 RND A
[15] 'CORRELATION R           = ';4 RND T[5;2]
[16] '2 VALUES OF X         = ';3 RND X[1,N]
[17] 'CORRESPONDING Y        = ';3 RND (B×X[1,N])+A
[18] 'KM                      = ';4 RND KM
[19] 'CONFIDENCE LIMITS      = ';4 RND (B+E)÷A;' TO ';
      4 RND (B-E)÷A
[20] 'VMAX                    = ';3 RND 1÷B
[21] 20 40 PLOT Q
      ∇

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WINHOW

FUNCTION WIN WILL DETERMINE VM, KM AND ITS 95 PERCENT CONFIDENCE LIMITS BY THE WILKINSON PLOT. X IS THE VECTOR FOR SUBSTRATE CONCENTRATIONS, AND V THE CORRESPONDING VELOCITIES. REQUIRES FUNCTIONS SR, RND (SMILLIE, 1969), AND PLOT (FALKOFF AND IVERSON, 1968). T-VALUES CAN BE CHANGED AT STATEMENT [9].


```

      ∇ LIN[ ] ∇
    ∇ S LIN V;T;Q;W;X;Y:A;B;KM;L;D;J;O;A;B;C;T2;E;X;TE
[1]  T←(5 3)ρ0
[2]  '1/S:                1/V: ';3 RND Q←Q(2,N←ρX)ρ(X←1÷S),(Y←1
      ÷V)
[3]  T←X SR Y
[4]  X←T[1;1]
[5]  KM←(B←T[4;1])÷A←T[3;1]
[6]  D←(+/(Y-(A+B×X))*2)÷J←N-2
[7]  L←D×(1÷N)+(X*2)÷W←+/(X-X)*2
[8]  O←(M←D÷W)×-X
[9]  TE← 0 4.303 3.182 2.776 2.571
      2.447 2.365 2.306 2.262 2.228
[10] A←(A*2)-(T2←TE[J]*2)×L
[11] B←(A×B)-T2×O
[12] C←(B*2)-T2×M
[13] E←((B*2)-A×C)*0.5
[14] 'Y                    = ';3 RND B;'X + ';3 RND A
[15] 'CORRELATION R        = ';4 RND T[5;2]
[16] '2 VALUES OF X      = ';3 RND X[1,N]
[17] 'CORRESPONDING Y      = ';3 RND(B×X[1,N])+A
[18] 'KM                    = ';4 RND KM
[19] 'CONFIDENCE LIMITS   = ';4 RND(B+E)÷A;' TO ';
      4 RND(B-E)÷A
[20] 'VMAX                  = ';3 RND 1÷A
[21] 20 40 PLOT Q
    ∇

```

LINHOW

FUNCTION LIN WILL DETERMINE VM, KM AND ITS 95 PERCENT CONFIDENCE LIMITS BY THE LINEWEAVER-BURK PLOT. S IS THE VECTOR FOR SUBSTRATE CONCENTRATIONS, AND V THE CORRESPONDING VELOCITIES. REQUIRES FUNCTIONS SR, RND (SMILLIE, 1969), AND PLOT (FALKOFF AND IVERSON, 1968). T-VALUES CAN BE CHANGED AT STATEMENT [9].


```

      V HILL[ ] V
    V VVM HILL S;V;VM;N;A;C;B;X1;Y1;D;R;SX;SY;SB;Z;Q;X;Y
[1]  VM+VVM[pVVM]
[2]  V←-1+VVM
[3]  N←pX+10⊗S
[4]  'LOG X:          LOG Y: ' ; 3 RND Q+Q(2,N)pX,(Y+10⊗V÷VM-V)
[5]  B←(A←(N×+/X×Y)-X1×Y1←+/Y)÷C←(N×+/X×X)-(X1←+/X)*
      2
[6]  R←((A×A)÷C×D←(N×+/Y×Y)-Y1*2)*0.5
[7]  SX←(C÷N×(N-1))*0.5
[8]  SY←(D÷N×(N-1))*0.5
[9]  SB←(SY÷SX)÷((N-2)÷(1-R×R))*0.5
[10] 'Y              = ' ; 3 RND B; 'X + ' ; 3 RND Z←(Y1÷N)-B
      ×X1÷N
[11] 'CORRELATION R   = ' ; 4 RND B
[12] '2 VALUES OF X   = ' ; 3 RND X[1,N]
[13] 'CORRESPONDING Y  = ' ; 3 RND (B×X[1,N])+Z
[14] 'HILL COEFFICIENT = ' ; 3 RND B; ' +- ' ; 3 RND SB
[15] 'APPARENT KM      = ' ; 3 RND 10*|Z÷B
[16] 20 40 PLOT Q
    V

```

HILLHOW

FUNCTION HILL WILL DETERMINE HILL COEFFICIENT (N) AND APPARENT KM BY THE HILL PLOT. VVM IS THE VECTOR FOR VELOCITIES WITH VM (THE MAXIMAL VELOCITY) AS THE LAST ELEMENT, AND S THE SUBSTRATE CONCENTRATIONS. REQUIRES FUNCTIONS RND (SMILLIE, 1969), AND PLOT (FALKOFF AND IVERSON, 1968).


```

      VDIX[ ]VD
      V I DIX V;R;N;SE;A;J;X;Y;D;B;Z;KI;X;Y;IN;M
[1]   J←1
[2]   '1/V:  ';3 RND M←1÷V
[3]   B←Z←SE←2ρ0
[4]   RE:X←I[J;]
[5]   Y←M[J;]
[6]   C←((N←(ρM)[2])×+/X*2)-(X←+/X)*2
[7]   B[J]←(A←(N×+/X×Y)-X×Y←+/Y)÷C
[8]   R←((A*2)÷C×D←(N×+/Y*2)-Y*2)*0.5
[9]   SE[J]←(((1-R*2)×D÷N)÷N-2)*0.5
[10]  '1/V = ';3 RND B[J];'I + ';3 RND Z[J]←(Y÷N)-B[J]×X÷N
[11]  'R      = ';3 RND R
[12]  '2 X = ';3 RND X[1,N]
[13]  '2 Y = ';3 RND(B[J]×X[1,N])+Z[J]
[14]  →(2≥J←J+1)/RE
[15]  'KI      = ';3 RND KI←(-/Z)÷-/B
[16]  →(((IN←(Z[1]+B[1]×-KI))-2×[/SE)>0)/POS
[17]  'NON-COMPETITIVE INHIBITION'
[18]  →21
[19]  POS:'COMPETITIVE INHIBITION'
[20]  'COMMON INTERCEPT AT Y  =';3 RND IN
[21]  20 40 PLOTQ(3,N)ρI[1;],M[1;],M[2;]
      V

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DIXHOW

FUNCTION DIX WILL DETERMINE KI, AND PATTERN OF INHIBITION BY THE DIXON PLOT. I IS A (2 × N) MATRIX OF INHIBITOR CONCENTRATIONS, AND V A (2 × N) MATRIX OF CORRESPONDING VELOCITIES. REQUIRES FUNCTIONS RND (SMILLIE, 1969), AND PLOT (FALKOFF AND IVERSON, 1968).


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      VTAK[[]]V
V VM TAK I;V;VI;N;A;C;B;X1;Y1;D;R;SX;SY;SB;Z;Q;X;Y
[1] V←1+VM
[2] VI←1+VM
[3] N←pX+10⊕I
[4] 'LOG X:          LOG Y:';3 RND Q←Q(2,N)pX,(Y←10⊕(V-VI)÷VI
    )
[5] B←(A←(N×+'X×Y)-X1×Y1←+,Y)÷C←(N×+/X×X)-(X1←+/X)*
    2
[6] R←((A×A)÷'×D←(N×+/Y×Y)-Y1*2)*0.5
[7] SX←(C÷N×(N-1))*0.5
[8] SY←(D÷N×(N-1))*0.5
[9] SB←(SY÷SX)÷((N-2)÷(1-R>R))*0.5
[10] 'Y              = ' ;3 RND B;'X + ' ;3 RND Z←(Y1÷N)-
    B×X1÷N
[11] 'CORRELATION R   = ' ;4 RND R
[12] '2 VALUES OF X = ' ;3 RND X[1,N]
[13] 'CORRESPONDING Y = ' ;3 RND(B×X[1,N])+Z
[14] 'HILL COEFFICIENT = ' ;3 RND B;' + - ' ;3 RND SB
[15] 'I50             = ' ;3 RND 10*|Z÷B
[16] 20 40 PLOT Q
V

```

TAKHOW

FUNCTION TAK WILL DETERMINE HILL COEFFICIENT (N) AND I50 BY THE HILL PLOT FOR INHIBITION STUDIES. VM IS THE VECTOR FOR VELOCITIES WITH VO (THE UNINHIBITED RATE) AS THE FIRST ELEMENT, AND I THE INHIBITOR CONCENTRATIONS. REQUIRES FUNCTIONS RND (SMILLIE, 1969), AND PLOT (FALCOFF AND IVERSON, 1969).


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      ▽CHAN[ ]▽
    ▽ CHAN;I;P;F;Y
[1]  'LIST INITIAL LIGAND CONCENTRATIONS PLEASE.'
[2]  I←[ ]
[3]  'LIST CPM FOR THE ENZYME CHAMBER PLEASE.'
[4]  P←[ ]
[5]  'LIST CPM FOR THE CHAMBER LESS ENZYME PLEASE.'
[6]  F←[ ]
[7]  CB←(I×P-F)÷P+F
[8]  CF←(I×F)÷P+F
[9]  ' '
[10] 'BOUND                FREE';3 RNDQ(2,ρI)ρCB,CF
[11] ' '
[12] 'DO YOU WANT THE SCATCHARD PLOT? YES OR NO PLEASE.'
[13] Y←[ ]
[14] →(Y[12]≠'YE')/0
[15] CB SCAT CF
    ▽

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CHANHOW

FUNCTION CHAN IS A CONVERSATIONAL PROGRAM THAT WILL CALCULATE BOUND AND UNBOUND LIGAND CONCENTRATIONS FOR EQUILIBRIUM DIALYSIS ACCORDING TO THE METHOD OF CHANGEUX, GERHART AND SCHACHMAN (1968). REQUIRES FUNCTIONS RND (SMILLIE, 1969), AND SCAT.


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      ▽ SCAT[ ] ▽
    ▽ X SCAT Y;PX;PY;A;P;Q
[1]  'WHAT IS THE AMOUNT OF PROTEIN?'
[2]  P←
[3]  PX←X÷P
[4]  PY←PX÷Y
[5]  A←(5 3)ρ0
[6]  A←PX SR PY
[7]  ' '
[8]  'R:                R/FREE:      ';Q←Q(2,ρX)ρPX,PY
[9]  ' '
[10] 'Y = ';4 RND A[4;1];'X + ';4 RND A[3;1]
[11] 'COEFFICIENT OF CORRELATION = ';4 RND A[5;2]
[12] 'DISSOCIATION CONSTANT      = ';4 RND 1÷|A[4;1]
[13] 'NUMBER OF SITES AVAILABLE  = ';4 RND A[3;1]÷|A[
      4;1]
[14] 20 40 PLOT Q
    ▽

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SCATHOW

FUNCTION SCAT WILL DETERMINE THE INTRINSIC DISSOCIATION CONSTANT (K), AND THE NUMBER OF BINDING SITES (N) BY THE SCATCHARD PLOT. X IS THE VECTOR FOR CONCENTRATIONS OF LIGAND BOUND TO PROTEIN, AND Y FOR CONCENTRAIONS OF LIGAND UNBOUND. REQUIRES FUNCTIONS SR AND RND (SMILLIE, 1969).

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